University of Alberta

Regulation of Acyl-CoA oxidase gene expression in loblolly pine (*Pinus taeda* L.) seeds following imbibition

By

Tito Migabo

A thesis submitted to Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Edmonton, Alberta

Fall, 2000

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Degree: Doctor of Philosophy

Year this degree was granted: 2000

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Abstract

Triacylglycerols (TAG), the major storage reserve in loblolly pine (*Pinus taeda* L.) seeds, are primarily located in the megagametophyte. Triacylglycerols break down to soluble carbohydrates following germination, and are imported by the growing seedling as a nutritional source. Triacylglycerol break down involves two glyoxysomal processes, β-oxidation and the glyoxylate cycle. Acyl-CoA oxidase (ACOX) is the enzyme that catalyzes the first and rate-limiting step of the β-oxidation spiral. Acyl-CoA oxidase transfers two electrons to FAD to form $FADH_2$, which is oxidized by O_2 to form H_2O_2 and FAD; the H_2O_2 is then dismutated by CAT to form molecular oxygen and water. Acyl-CoA oxidase was purified to homogeneity from loblolly pine, to generate biochemical and molecular tools to study the regulation of TAG breakdown. The enzyme was shown to be a homodimer with a native molecular mass of 150 kD and a subunit molecular mass of 71 kD. Polyclonal antibodies raised in rabbits were shown to be monospecific for ACOX, by immunotitration, western blotting and immunoprecipitation. Acyl-CoA oxidase antibodies were used to screen a loblolly pine expression library and isolate a cDNA clone, which had a deduced amino acid sequence that showed high similarity (75%) with equivalent sequences from other plant and animal sources. The sequence contained a PSTI type peroxisomal targeting motif and a FAD binding motif. Western and northern blot analyses showed that ACOX gene expression and protein production was developmentally regulated; regulation of gene expression was at the level of mRNA. Maximum rate of megagametophyte TAG breakdown and ACOX

enzyme activity was only achieved if the developing seedling was present to act as a sink for carbohydrate produced. Seedling removal resulted in a decrease in megagametophyte ACOX protein and mRNA. It is possible that ACOX gene expression is under the regulation of carbon catabolite repression at the level of mRNA.

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Regulation of Acyl-CoA oxidase gene expression in loblolly pine (*Pinus taeda* L.) seeds following imbibition" submitted by Tito Migabo in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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To my Family

Acknowledgements

I would like to thank my supervisor, Dr. Gifford, my supervisory committee members, Dr. Taylor and Dr. Ozga for their help throughout my program.

I would also like to thank my colleagues: members of Dr. Gifford's lab (past and present) and members of Dr. Taylor's lab (past and present) for their help, company and encouragement.

Table of contents

Chapter	Page
1. Introduction	1
1.1 Loblolly pine	1
1.2 Dormancy and germination	3
1.3 Changes in patterns of gene expression and early seedling growth	5
1.4 Seed storage reserves	7
1.4.1 The seed storage proteins	7
1.4.2 Storage protein synthesis	9
1.4.3 The seed storage lipids	10
1.4.4 Synthesis of Triacylglycerol	11
1.5 Protein reserve breakdown	14
1.6 Peroxisomes	16
1.7 Translocation of proteins into peroxisomes	17
1.8 TAG breakdown	18
1.9 Regulation of TAG breakdown	22
1.10 Effect of embryo on reserve mobilization	23
1.11 The present study	24
2. Materials and Methods	26
2.1 Seed material and germination	26
2.2 Chemicals and equipment	27
2.3 Protein and enzyme assays	28
2.3.1 Protein assay	28

2.3.2 Catalase assay 29

2.3.3 Acyl-CoA oxidase assay 29

2.4 Purification of ACOX 30

2.5 Molecular mass determination 31

2.6 ACOX antibody production 32

2.7 *In vivo* protein labeling 33

2.8 Electrophoresis 33

2.8.1 SDS-PAGE 33

2.8.2 2D-SDS-PAGE 34

2.8.3 Isoelectric focusing 35

2.9 Western Transfer 35

2.10 Immunotitration 36

2.11 Immunoprecipitation 36

2.12 Immunoscreening of cDNA library with ACOX antibody 38

2.13 DNA sequencing 39

2.14 RNA isolation and northern blot analysis 40

2.15 Determination of Triacylglycerols 41

2.16 Carbohydrate determination 42

3. Results 43

3.1 ACOX activity in megagametophytes 43

3.2 ACOX enzyme purification 44

3.3 Immunological characterization of ACOX 56

3.4 Tissue distribution of ACOX protein 56

3.5 Determination of ACOX pI 63

3.6 Isolation and charaterization of an ACOX cDNA 63

3.7 A comparison of loblolly pine ACOX deduced amino acid sequence with ACOX amino acid sequences from other species 64

3.8 Tissue distribution of ACOX41 transcripts 69

3.9 Changes in ACOX protein levels *in vivo* 69

3.10 *De novo* ACOX protein synthesis 72

3.11 Changes in ACOX transcripts during megagametophyte development 72

3.12 Effect of embryo removal on in vivo protein synthesis in the developing megagametophytes 75

3.13 Effect of embryo removal on TAG concentrations in the megagametophyte during germination and post germinative growth 76

3.14 Effect of embryo removal on carbohydrate levels in the megagametophyte during germination and post germinative growth 83

3.15 Effect of embryo removal on ACOX and CAT activities 88

3.16 Effect of soluble carbohydrates on ACOX activity *in vitro* 88

3.17 Effect of embryo removal on the accumulation of ACOX protein in megagametophytes cultured *in vitro* 93

3.18 Effect of embryo removal on the accumulation of ACOX transcripts in megagametophytes cultured *in vitro* 93

4. Discussion 96

4.1 Purification and characterization of ACOX 96

4.2 Isolation of a cDNA that encodes ACOX 98

4.3 ACOX synthesis and expression: changes occurring during germination and early seedling growth 100

4.3.1 Tissue specificity 100

4.3.2 Temporal changes following seed imbibition 102

4.4 ACOX synthesis and expression: the relationship between the megagametophyte and embryo 103

4.4.1 TAG breakdown 103

4.4.2 ACOX gene expression and enzyme activity 107

4.5 Future studies 109

5. Literature cited 110

List of tables

Table	Page
Table 1 Purification procedure of ACOX	55

List of figures

Figure		Page
Figure 1.1	Natural range of loblolly pine	2
Figure 1.2	Triacylglycerol biosynthesis pathway	12
Figure 1.3	Direction of movement of breakdown products in a loblolly pine seed following germination	20
Figure 1.4	Triacylglycerol breakdown pathway	21
Figure 3.1	Changes in ACOX activity during megagametophyte development	46
Figure 3.2	Fractionation of proteins by hydrophobic chromatography	48
Figure 3.3	Separation of ACOX on hydroxyapatite column	50
Figure 3.4	Purification ACOX on Ultrogel AcA-34	52
Figure 3.5	Progress of purification of ACOX shown by gel electrophoretic analysis	54
Figure 3.6	Native molecular mass determination of ACOX	58
Figure 3.7	Immunotitration of ACOX activity	60
Figure 3.8	Tissue distribution of ACOX protein	62
Figure 3.9	Nucleotide and deduced amino acid sequences of ACOX	66
Figure 3.10	Alignment of amino acid sequences of loblolly pine ACOX, arabidopsis ACOX, barley ACOX and rat ACOX	68
Figure 3.11	Tissue distribution of ACOX41 transcripts	70
Figure 3.12	Changes in ACOX steady state protein levels during megagametophyte development	71
Figure 3.13	*De novo* synthesis of ACOX protein	73
Figure 3.14	Accumulation of ACOX mRNA during megagametophyte development	74

Figure 3.15 *In vivo* protein synthesis as shown by one-dimensional gel electrophoresis 78

Figure 3.16 *In vivo* protein synthesis as shown by two-dimensional gel elctrophoresis 80

Figure 3.17 Quantitative changes of TAG levels in megagametophytes cultured *in vitro* with or without embryos 82

Figure 3.18 Quantitative changes of soluble carbohydrate levels in megagametophytes cultured *in vitro* with or without embryos 85

Figure 3.19 Quantitative changes of insoluble carbohydrate levels in megagametophytes cultured *in vitro* with or without embryos 87

Figure 3.20 Effect of embryo removal on ACOX activity in megagametophytes cultured *in vitro* with or without embryos 90

Figure 3.21 Effect of embryo removal on CAT activity in megagametophytes cultured *in vitro* with or without embryos 92

Figure 3.23 Effect of embryo removal on ACOX protein in megagametophytes cultured *in vitro* with or without embryos 94

Figure 3.24 Effect of embryo removal on ACOX41 transcripts in megagametophytes cultured *in vitro* with or without embryos 95

List of abbreviations

ABA	abscisic acid
ACOX	acyl-CoA oxidase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp; kb	base pair; kilobase
Bq	becquerel
BSA	bovin serum albumin
CAT	catalase
cDNA	complementary DNA
µCi	microcurie
CoA	coenzyme A
Da; kD	Dalton; kilodalton
DAI_{30}	days after imbibition at 30^0C
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
EDTA	ethylenediaminetetraacetic acid
HTP	hydroxyapatite
ICL	isocitrate lyase
IEF	isoelectric focusing
IPTG	isopropyl β-D-Thiogalactoside
Kat	katal
MS	malate synthase

MOPS	3-(N-Morpholino)propanesulphonic acid
mRNA	messenger ribonucleic acid
NBT	nitroblue tetrazolium
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PI	isoelectric point
PTS	peroxisomal targeting signal
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
TAG	triacylglyerol
TBS	Tris buffered saline
TCA	trichloroacetic acid

1 Introduction

1.1 Loblolly Pine

Loblolly pine (*Pinus taeda* L.) is the leading timber species in the United States. This tree species covers a total area of more than 13.4 million hectares of southern forest lands, which is more than half the total volume of southern pine growing stock; other economically important southern pines include long leaf (*Pinus palustris* Mill., short leaf (*Pinus echinata* Mill., and slash pine (*Pinus elliottii* Engelm.)(Schultz, 1997). The total biomass in loblolly pine exceeds one million metric tons dry weight (Cost, et al., 1990). Loblolly pine is a large tree averaging 27 to 34 meters in height and 61 to 76 centimeters in diameter at a mature age of between 80 to 100 years (Schultz 1997). It is the most versatile of all southern pines and can grow on diverse ecological sites. In the United States loblolly pine is found naturally in fifteen southern and mid Atlantic states. Its natural range extends from Delaware, New Jersey and eastern Maryland in the north to central Florida in the south. To the south west, the range extends to eastern Texas, the south eastern part of Oklahoma and southern Arkansas (figure 1.1, Schultz 1997). Loblolly pine's importance as a soft wood species stems from its wood properties, genetic variability, and quick regeneration and rapid growth. In the United States it is mainly used in the pulp and paper industry.

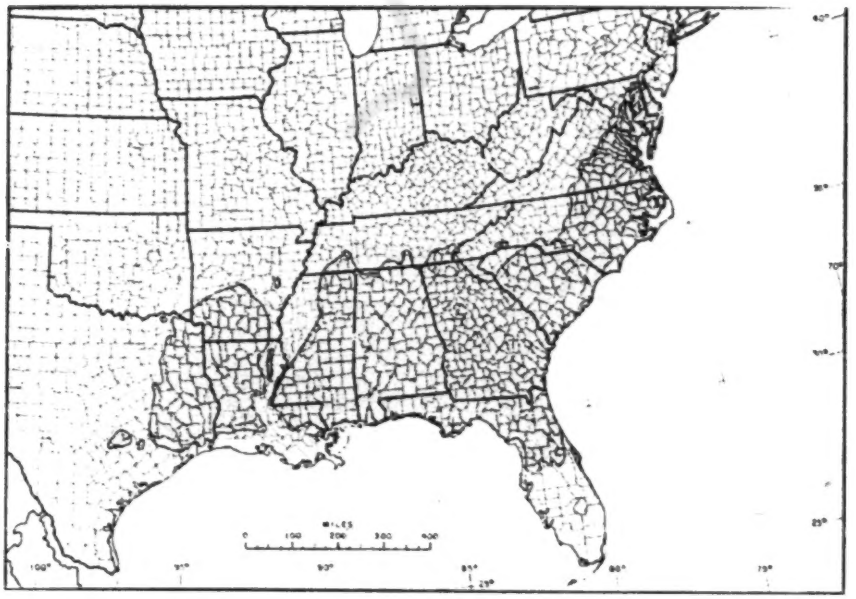


Figure 1.1. Natural range of loblolly pine is shown in dark gray. Taken from Schultz, 1997

Scientifically, loblolly pine is one of the most intensively studied forest tree species in the world. The initial studies of loblolly pine related to tree improvement, specifically aimed at altering wood quality (Kellison and Gingrich, 1982). Because germination rates and seedling vigor are generally poor (Edwards 1986), several biochemical and physiological studies of loblolly pine seeds have been initiated to try and identify processes that may improve germinability. In relation to these, chemical treatments have been developed to improve germination of recalcitrant loblolly pine seeds (Hare 1981).

1.2 Dormancy and Germination

Seeds that exhibit dormancy fail to germinate when exposed to environmental conditions that are favorable for germination. Seed dormancy can be due to physical or physiological blocks (Bewley, 1997). Physical blocks are often due to constraints imposed by the seed coat. Physiological factors that block germination may reside in the embryo itself. Abscisic acid (ABA) has been implicated as a major dormancy-imposing factor in many species (Bewley, 1997). Compared to other southern pines, loblolly pine seeds are the most dormant (Pawuk and Barnett 1979). Under natural conditions, loblolly pine seeds are dispersed in the fall and stay on the forest floor for about four months during which time the seeds are exposed to cold, moist conditions which break seed dormancy; germination then occurs in the spring (Schultz, 1997). This prechilling

(also called stratification) not only overcomes seed dormancy but also makes germination rapid and uniform, and promotes seedling survival and uniform seedling establishment (Schultz, 1997). Under laboratory conditions, loblolly pine seeds usually require a stratification period of about 35 days at 2^0C. Schneider and Gifford (1994), suggested that in loblolly pine, the seed coat is the primary cause of dormancy. It causes a mechanical constraint that prevents radicle expansion; it is this constraint that is overcome by stratification (Carpita, et al., 1983; Gifford, unpublished data).

For non-dormant mature desiccated seeds, germination begins with water uptake and ends with radicle emergence (Bewley and Black 1994). For dormant seeds such as loblolly pine, water uptake occurs during stratification and stratified seeds will germinate when placed at a suitable temperature (~30^0). At the onset of germination, the rate of cellular metabolism increases rapidly; this includes respiration, and protein and RNA synthesis (Bewley and Black 1994). The biochemical and molecular processes that follow the onset of germination have mainly been studied in angiosperms (Bewley and Black, 1994; Thomas, 1993; Bewley and Marcus, 1990). In this introduction, I will focus on changes in patterns of gene expression following the onset of germination in conifers in relation to storage reserve breakdown.

1.3 Changes in patterns of gene expression during germination and early seedling growth

Biochemical and molecular events that take place following the onset of germination depend on proteins and mRNAs that are present in the mature desiccated seed (Bewley and Black, 1994; Payne, 1976). These proteins and mRNAs are synthesized during maturation phase of seed development. The preformed mRNAs encode proteins that are required to initiate germination (Whitmore, 1991); they decay rapidly once germination has started (Aspart et al., 1984; Thompson and Lane, 1980). Newly transcribed mRNA populations have also been identified following the onset of germination (Bewley et al., 1989; Litts et al., 1987; Grzelczak et al., 1982; Peumans et al., 1982; Thompson and Lane, 1980). Conifers are similar in this regard. Masumori et al. (1992), using RNA blot hybridization techniques quantified different stored RNA species in desiccated *Pinus thunbergii* seeds that had been preserved for at least 14 years. This group performed Northern blot hybridizations using cDNA probes generated from stored mRNAs and showed that some of the mRNAs disappeared immediately following the onset of germination while others persisted and were detected at later stages of germination and into post germinative growth. In loblolly pine, Mullen et al., (1996) analyzed mRNA populations in the mature seed, during stratification, germination and post germinative growth, and showed that distinct populations of mRNAs are developmentally regulated in both the megagametophyte and the embryo. These alterations in gene expression are in a

temporal fashion that is consistent with the patterns of *de novo* protein synthesis previously shown for lodgepole pine (Gifford et al., 1991) and loblolly pine (Groome et al., 1991). Also, *de novo* synthesis of proteins occurs during stratification of loblolly pine seeds (Schneider and Gifford 1994). According to Gifford et al. (1991), three patterns of protein synthesis can be identified; the first generates a group of constitutively synthesized proteins; the second produces a group, which is found in mature seed, and is only synthesized during early germination; the third synthesized a group that appears following the completion of germination during post germinative growth. Stabel, et al. (1990) showed similar patterns of protein synthesis in a study of Norway spruce. Although these groups of proteins were not biochemically characterized in terms of their functions, Gifford et al. (1991) proposed that the first group of proteins is used for maintenance of the general cellular metabolism, the second group is for germination associated processes and the third group contains enzymes involved in the breakdown of seed storage reserves.

Sandas, et al. (1992) showed that in Norway spruce embryos, *de novo* protein synthesis during germination and post germinative growth correlated with the expression of specific cDNAs. Similar results were found in imbibed Douglas-fir seeds (Taylor and Davies, 1995). Tranbarger and Misra (1996) further characterized the pattern of gene expression in conifers during these developmental periods. Using cDNAs from a Douglas fir germination specific cDNA library, they performed differential Northern blot analysis, and showed that these cDNAs code for proteins that are involved in biochemical and physiological

changes that take place during this time in the conifer life cycle. Collectively, all these studies suggest that gene expression follows a specific pattern during germination and early seedling growth.

1.4 Seed Storage Reserves

In conifers, the major seed storage reserves are proteins and lipids and are primarily localized within in the haploid megagametophyte, in specialized subcellular organelles known as protein and lipid bodies (Groome et al., 1991; Misra and Green, 1990; Gifford and Tolley, 1989; Gifford, 1988; Salmia, 1981; Ching, 1966). These reserves are laid down during seed development to be used later during and following germination, when they will support protein synthesis and production of energy for early seedling establishment until the seedling becomes fully autotrophic (Bewley and Black, 1994; Derbyshire et al., 1976).

1.4.1 The seed storage proteins

Osborne (1916) divided the seed storage proteins into four classes based on their solubilities.

1-albumins, which are soluble water and dilute buffers at neutral pH

2-globulins, which are which are insoluble in water but soluble in salt solutions

3-glutelins, which are soluble in acid and alkali solutions

4-prolamins, which are alcohol soluble

This classification is not ideal, especially when current research uses different extraction procedures. Storage proteins can also be characterized by their sedimentation coefficients (S values). Most albumins are referred as 2S albumins and are small, globular, heterodimeric proteins consisting of a large and small subunit linked by disulfide bridges (Shewry et al., 1995). Because, some 2S storage proteins show globulin-like solubilities and yet share structural features with 2S albumins, Allona et al., (1994a) consider both 2S albumins and 2S globulins as a single class of 2S proteins. Globulins constitute a class of 11S proteins that are often referred to as legumin-like proteins and another class of 7S which are also called vicilin-like proteins (Shewry et al., 1995; Higgins, 1984). The 11S globulins are hexameric proteins, with 50-55 kD subunits. Each of these subunits consists of an acidic (30-34 kD) and basic (20-23 kD) subunit polypeptide and these are linked by a disulfide bridge (Bewley and Black, 1994; Allona et al., 1992; Groome et al., 1991; Jensen and Lixue, 1991; Lammer and Gifford, 1989; Gifford, 1988). The 7S vicilin-like are heterotrimeric proteins that do not form disulfide bridges (Shewry et al., 1995). Another class of storage proteins known as crystalloids (glutelin-like, 11S globulins) which are found in castor bean and many conifer species, have minimum solubility in salt solutions, minimum solubility in acid and alkali solutions but have complete solubility in

solutions containing chaotropic agents such as SDS and urea (Bewley and Black, 1994). Crystalloid proteins have been found in species of *Pinus* (Jensen and Lixue 1991; Gifford 1988,) and *Picea* (Misra and Green 1991; Hakman et al., 1990, Misra and Green 1990; Gifford and Tolley 1989). Glutelins are restricted to cereals and completely soluble in solutions containing chaotropic agents such as urea and SDS. They consist of high and low molecular weight subunits that are held together disulfide bridging (Casey and Domoney 1987). Prolamins are the major storage proteins in most cereals (Shrewry et al., 1995). They may be either S-rich proteins or S-poor proteins. The S-rich proteins have extensive disulfide bridges and therefore have a tendency of being polymeric whereas the S-poor proteins are not oligomeric because they lack cysteine residues. In either case, these proteins are mainly rich in proline and glutamine residues that are usually in repeated sequences (Shewry et al., 1995).

1.4.2 Storage protein synthesis

The major site for storage protein synthesis is in the cytoplasm on polysomes that are associated with the rough endoplasmic reticulum (RER). Initially, the mRNA is translated to produce a protein precursor that has a signal peptide with a hydrophobic core, which enables the protein to be sequestered into the lumen of the RER. The signal peptide is removed in the ER by a signal peptidase. The protein precursors form aggregates due to their hydrophobic nature and these

aggregates move into the Golgi apparatus via cytoplasmic streaming, where post-translational modifications may occur. The protein precursors are sorted into transfer vesicles at the trans-Golgi network. The vesicles are then targeted into storage protein bodies where further modification occurs (Bewley and Black 1994; Muntz et al., 1994; Muntz et al., 1985). These modifications usually involve endopeptidase cleavage to form subunit polypeptides. Sometimes, further modifications occur at the C-terminal of these polypeptides. For example Staswick et al. (1984), showed that additional residues were removed prior to subunit formation.

1.4.3 The seed storage lipids

Lipids are defined as substances of biological origin that are soluble in organic solvents such as chloroform but are insoluble in water (Alberts et al., 1994). Most plant and animal storage lipids are fatty acid esters of glycerol more commonly called triacylglycerols (TAGs). The main seed storage reserves in Pinaceae plants are TAGs (Hammer and Murphy, 1994; Ching, 1966) where they make up 58% of the total seed reserve (Stone and Gifford 1997). TAGs are high energy reservoirs, containing more than twice the energy of protein or starch per unit weight (Huang 1992). Fatty acids are identified by the number of carbons they contain, the double bonds in their hydrocarbon chains and the positions where the double bonds are located in their chains. Over 90% of fatty acids in

TAGs of Pinaceae are unsaturated (Wolff et al., 1997, Imbs and Pham, 1996; Janick et al., 1991). TAGS of most oil seeds contain oleic (18:1), linoleic (18:2) and linolenic (18:3) acids among other fatty acids. The common positions of these double bonds are 18:1 (cis Δ9), 18:2 (cis Δ9, Δ12) and 18:3(cis Δ9, Δ12, Δ15). However, conifer TAGs have unique fatty acids with double bonds at position carbon number five, for example 18:3 (cis Δ5, Δ9, Δ12) is only found in conifers (Imbs and Pham, 1996, Janick et al., 1991).

1.4.4 Synthesis of triacylglycerol

The main steps in TAG biosynthesis are the production of glycerol backbone, the formation of fatty acids and the esterification of glycerol with different fatty acid components to produce TAGs (Alberts et al., 1994; Bewley and Black, 1994). A general pathway for TAG biosynthesis is shown in figure 1.2. During seed development, sucrose is converted into hexose and triose phosphates in the glycolytic pathway. The triose phosphates are reduced to *sn*-3 glycerol-3-phosphates, which become the glycerol backbone. Most of fatty acid synthesis occurs in the plastid (Bewley and Black, 1994; Somerville and Browse, 1991). The starting substrate for fatty acid synthesis is acetyl-CoA, which is carboxylated to yield malonyl-CoA. The malonyl residue of the malonyl-CoA is then transferred to acyl carrier protein (ACP) to form malonyl-ACP. A condensation reaction between acetyl-CoA and malonyl moieties forms acetoacetyl-ACP with a

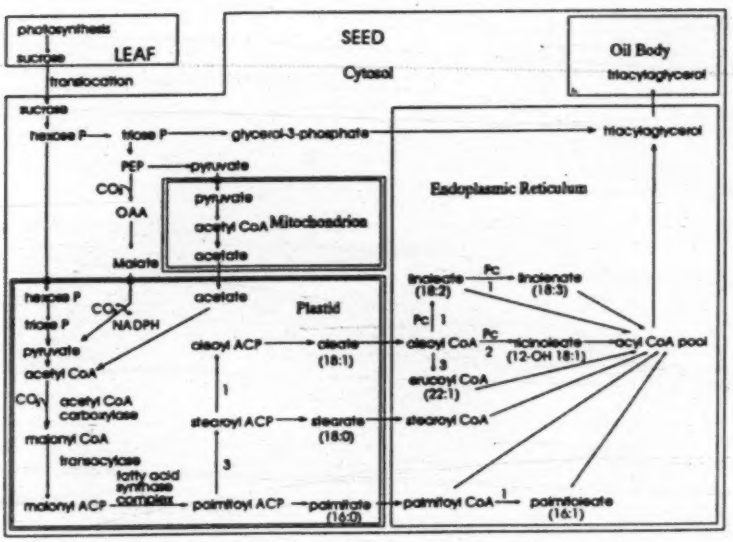


Figure 1.2. Scheme for the synthesis of fatty acids in seeds. Synthesis involves three cell compartments: the cytosol, the plastid, and the endoplasmic reticulum, the latter becoming modified to form the oil bodies. Numbered steps require the following enzymes:
1, desaturase; 2, hydrolase; 3, elongase. Polyunsaturation and hydroxylation takes place on acyl groups incorporated temporarily into phosphatidylcholine (PC).

Taken from Bewley and Black 1994

loss of CO_2. Two successive reduction steps, using NADPH and NADH and a dehydration converts acetoacetyl-ACP to a 4 carbon acyl residue. Successive condensations with further malonyl moieties, gives rise to a 6 carbon fatty acyl moiety. This sequential addition of 2C units continues to make a chain of 16C. The fatty acid dissociates from the fatty acid synthase complex, the enzymes responsible for these condensations, to yield palmitic acid esterified to an acyl carrier protein (16:0, palmitoyl-ACP). Further elongation of palmitoyl-ACP requires the presence of palmitoyl elongase, which converts palmitoyl-ACP to stearol ACP, which is an 18 carbon saturated fatty acid (18:0-ACP). Stearoyl ACP desaturase rapidly and efficiently converts 18:0-ACP to 18:1-ACP. Usually the first double bond is introduced at carbon 9 of stearic acid to yield oleoyl-ACP, but in the case of conifers the first double bond is introduced at carbon number five. The final reaction in the fatty acid synthesis is transacylation of oleoyl-ACP to oleoyl-CoA, which accumulates in the plastids. Oleoyl-CoA is the central metabolite in plant lipid metabolism. Oleoyl-CoA modifications, which occur on the ER, include further elongation, further desaturation, insertion of functional groups like OH moieties, or could be incorporated directly into glycerolipids in plastids (Somerville and Browse, 1991).

In the ER, a condensation reaction involving acyl groups and *sn*-3 glycerol-3-phosphate yields TAGs. Fatty acid esterification to the *sn*-1 and *sn*-2 positions on the glycerol backbone, is catalyzed by two different acyltransferases in two successive reactions, while diacylglycerol acyltransferase catalyzes the fatty acid esterification on the *sn*-3 position. The accumulation of the newly formed TAGs

between the two layers of the ER double membrane, causes the ER to swell, forming a vesicle which buds off and deposited as a lipid body. Since the TAGs accumulate in the membrane between the phospholipid layers, the lipid body is surrounded by a half-unit membrane.

The lipid body half-unit membrane is stabilized by a unique class of low molecular weight proteins (15-26 kD) called oleosins, which are structurally incorporated into the membrane. Oleosins have three structral domains: a) an amphipathic domain of 40-60 amino acids near the NH_2-terminal end, which is associated with the oil body surface; b) a hydrophobic domain of 68-74 amino acids that penetrates into the lipid body and lies within the TAG matrix; and c) an amphipathic alpha helical domain of 33-40 amino acids situated at or near the COOH-terminus, which also protrudes from the oil body surface. These oleosins impart an overall negative charge on the surface of the oil bodies, preventing the lipid bodies from coalescing and aggregating (Leprince, et al 1998; Bewley and Black 1994; Murphy, 1993; Tzen et al. 1993; Huang, 1992). Oleosins may also provide a binding site for lipases involved in TAG breakdown following germination (Bewley and Black 1994).

1.5 Protein reserve breakdown

Patterns of storage protein hydrolysis have also been described qualitatively in several conifer species that include white spruce (Gifford and Tolley, 1989),

lodgepole pine (Lammer and Gifford, 1989), Douglas-fir (Green, et al., 1991), loblolly pine (Groome, et al., 1991), and Scots pine (Salmia, et al., 1978)

Following germination, conifer seed storage proteins are hydrolyzed to small peptides and soluble amino acids, which are then transported to the seedling (King and Gifford; 1997, Lammer and Gifford, 1989). It is believed that in conifers storage protein reserves are broken down by endopeptidases (Salmia 1981) into soluble peptides, which are then subjected to the activity of aminopeptidases and carboxypeptidases to produce soluble amino acids (Gifford et al., 1989; Gifford and Tolley, 1989; Kovac and Kregar, 1989). The soluble amino acids are used by the developing seedling for energy production and for the synthesis of nucleotides and proteins.

Many studies of different plants suggest that endopeptidase activity is a necessary prerequisite for rapid breakdown of reserve proteins, which follows germination (Migabo, 1995; Akasofu, et al., 1989; Mitsuhashi and Minamikawa, 1989; Mitsuhashi, et al., 1986). For example the activity of an endopeptidase (EP-C1), but not exopeptidases, was found to increase continually throughout post germination and subsequent senescence of the pods of the *Phaseolus vulgaris* plants (Tanaka, et al., 1989; Endo, et al., 1987). Chrispeels and Boulter (1975) found that a sulfhydryl-type endopeptidase activity was responsible for initial hydrolysis of reserve proteins in mung beans.

1.6 Peroxisomes

Peroxisomes are members of the microbody family. Glyoxysomes found in plants, leaf-type peroxisomes and the glycosomes of trypanosomes are specialized type of peroxisomes. By definition, microbodies contain at least one hydrogen peroxide-producing oxidase, and catalase to decompose the hydrogen peroxide into water and molecular oxygen. These organelles appear spherical in electron micrograph, with diameters ranging from 0.2 µm to 1.5 µm (Subramani, 1993; van den Bosch, et al. 1992; Frederick, et al. 1974; Briedenbach and Beevers, 1967). Peroxisomes are delimited by a single unit membrane, which encloses a fine granular matrix that sometimes contains a paracrystalline core. Many biochemical functions take place in peroxisomes and these functions vary depending on the organism and cell type. Some of these functions include, fatty acid ß-oxidation, cholesterol metabolism, acetyl CoA metabolism via the glyoxylate cycle, alcohol oxidation, degradation of hydrogen peroxide, bile acid synthesis, photorespiration and plasmalogen synthesis (Gietl, 1996; Olsen and Harada, 1995; Stanway, et al. 1995; Subramani, 1993). Peroxisomes do not contain DNA, and do not contain any independent protein synthesis machinery as do mitochondria and chloroplasts and therefore all peroxisomal proteins are encoded by nuclear genes. Both soluble matrix and membrane bound peroxisomal proteins are synthesized on free polyribosomes in the cytosol, usually without precursor extensions and then translocated to the peroxisomes

(Olsen and Harada, 1995; van den Bosch, et al. 1992). ATP hydrolysis seems to be necessary for protein translocation into peroxisomes.

Leaf-type peroxisomes, microbodies in green leaves of higher plants, are the largest so far observed, often greater than 1.5 µm in diameter. These organelles, along with chloroplasts and mitochondria are the most numerous in mature photosynthetic cells (Frederick, et al. 1975). In addition to large amounts of catalase, these peroxisomes contain enzymes essential for reactions of photorespiration which include glycolate oxidase, a hydrogen peroxide-producing, flavin-linked oxidase, hydroxypyruvate reductase, and other enzymes involved in glycolate pathway (Subramani, 1993; Huang, et al. 1983; Tolbert, 1981; Frederick, et al. 1975).

Glyoxysomes contain both ß-oxidation and glyoxylate cycles which participate in the breakdown of storage fatty acids to acetyl-CoA and its subsequent conversion to succinate (Mittendorf, et al. 1999; Huang et al. 1983).

1.7 Translocation of proteins into peroxisomes

While compartmentalization of biochemical functions permits a level of metabolic control in eukaryotic cells, it presents the cell with the problem of targeting proteins from their sites of synthesis in the cytosol to their specific organellar locations (Olsen and Harada, 1995; Subramani, 1993; Lazarow and Fujiki, 1985). This targeting displays two levels of specificity: 1) the level of a

targeting motif within the protein, and 2) the level of receptors that recognize this motif as being specific for this particular compartment. There are multiple peroxisomal targeting signals (PTSs), that can be found located on different regions of peroxisomal proteins (Subramani, 1993). The most widely characterized, are located at the C-terminal. These are called PTS1 and are comprised of a three amino acid motif (SKL and its functional variants). This motif is the major targeting signal for proteins destined for the peroxisomal matrix. A second targeting motif is called PTS2, which is a conserved N-terminal nonapeptide (R/K)(L/V/I)$(X)_5$(H/Q)(L/A). This motif is used by a small subset of peroxisomal matrix proteins (Rachubinski and Subramani, 1995). There are other internally located PTSs that have been described but not fully characterized (Purdue and Lazarow, 1994). For example acyl-CoA oxidase from *Candida tropicalis*, possesses an internal targeting signal that does not resemble PTS1 or PTS2 (Gietl, 1996, Kamiryo, et al., 1989, Small, et al., 1988). Different import pathways for peroxisomal proteins with different PTSs have been studied, using mutations in PTS1 and PTS2 receptors (Brikner, et al., 1997; McNew and Goodman, 1996; Rachubinski and Subramani, 1995).

1.8 TAG breakdown

In conifer seeds, the majority of TAGs are located in the megagametophyte. Following germination, these TAGs are broken down to

produce carbohydrates that are rapidly exported to the developing seedling where they play a role in seedling establishment before autotrophy is fully achieved (Stone, 1999; Groome, et al., 1991; Ching, 1966). This is illustrated in figure 1.3. The TAG breakdown pathway is shown in figure 1.4. The TAGs are converted to glycerol and fatty acids by lipases, which are associated with lipid bodies (Hammer and Murphy, 1994). The glycerol enters the glycolytic pathway to produce triose phosphates, which may be condensed to yield hexose units, or converted to pyruvate that is then oxidized through the TCA cycle (Bewley and Black, 1994). Fatty acids are metabolized in glyoxysomes via reactions of β-oxidation to produce acetyl-CoA units that are used in the glyoxylate cycle to produce succinate. The succinate is transported to the mitochondrion where it is, in a series of reactions converted to oxaloacetate. The oxaloacetate is exported to the cytosol where it is converted to sucrose via gluconeogenesis (Figure 1.4, Gietl, 1992). Glyoxysomal enzymes that play a major role in these processes are acyl-CoA oxidase EC 1.3.3.6 (ACOX), catalase (CAT), isocitrate lyase (ICL) and malate synthase (MS). Following germination of oil seeds, the activities of enzymes of ß-oxidation and the glyoxylate cycle increase rapidly. But when photosynthesis is established, these enzyme activities diminish greatly. However, reactivation of ß-oxidation and the glyoxylate cycle enzyme activities have been reported in senescent organs, where they are believed to be involved in breakdown of membrane lipids (Graham, et al., 1992; De Bellis and Nishimura, 1991; Tolbert, 1981).

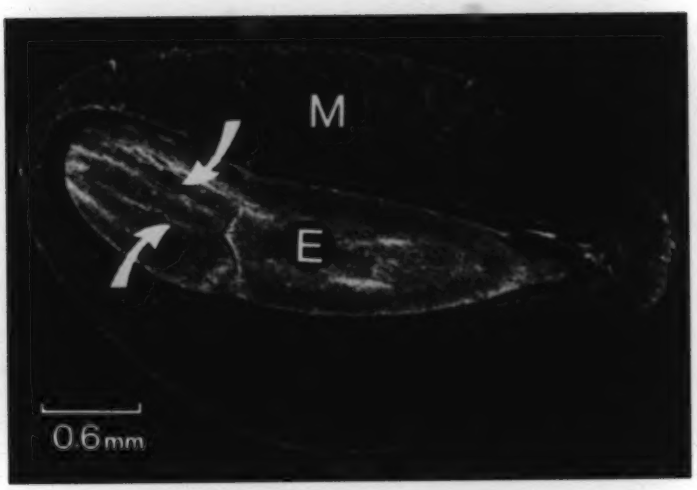


Figure 1.3. A longitudinal section through a loblolly pine seed with the seed coat removed, showing the megagametophyte (M) and the embryo (E). The arrows indicate the proposed direction of movement of storage reserve breakdown products.

Taken from Stone 1999.

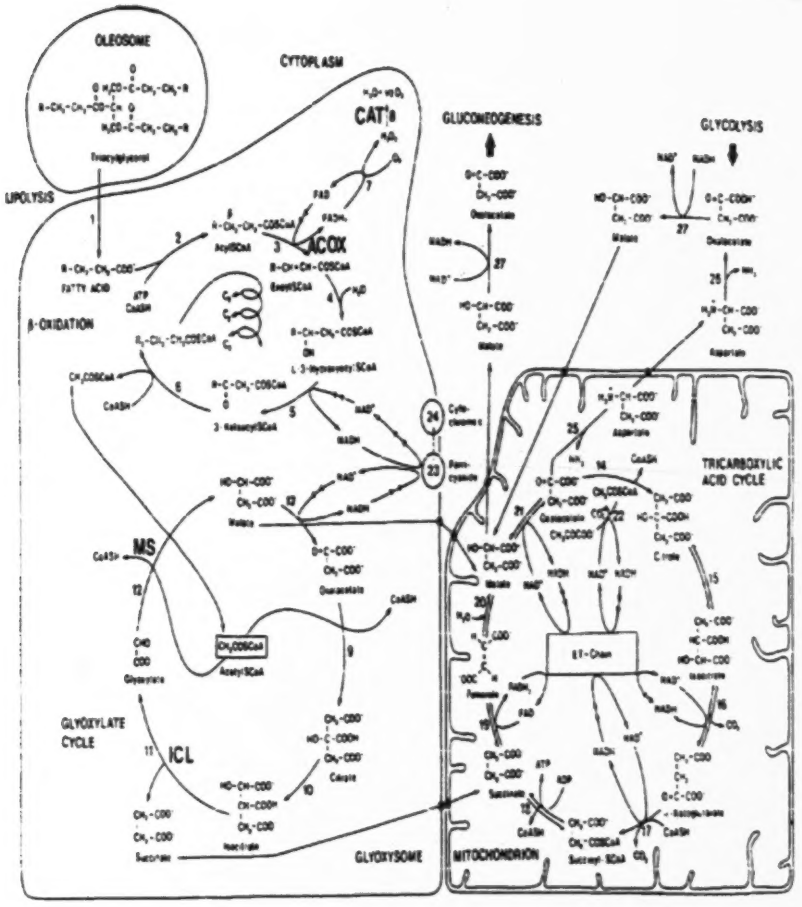


Figure 1.4 Flow of metabolites among the glyoxysome, mitochondrion and cytoplasm. Enzymes are: 1, lipase; 2, thiokinase; 3, acyl-CoA oxidase; 4 enolylhydratase; 5, hydroxyacyl dehydrogenase; 6, ketoacyl thiolase; 7, flavin oxidase; 8, catalase; 9, citrate sythase; 10, aconitase; 11, isocitrate lyase; 12, malate sythase; 13, malate dehydrogenase *g*; 14 citrate synthase; 15, aconitase; 16, isocitrate dehydrogenase, 17, α-ketoglutarte dehydrogenase; 18 succinate thiokinase; 19, succinate dehydrogenase; 20, fumarase; 21, malate dehydrogenase m; 22, pyruvate dehydrogenase; 23, ferricyanide reductase; 24 cytocrome b_5; 25, aspartate aminotransferase m; 26, aspartate aminotransferase c; 27, malate dehydrogenase *c*.

Taken from Gietl, (1992)

1.9 Regulation of TAG breakdown

The first step of the β-oxidation spiral is a reaction catalyzed by ACOX. This step has been shown to be rate limiting in cucumber (Kindl, 1987; Kirsch, et al., 1986), rat liver (Yukio and Lazarow, 1985; Osumi and Hashimoto, 1979), *Candida tropicalis* (Wayne and Rachubinski, 1987; Yukio et al., 1986), *Saccharomyces cerevisiae* (Luo, et al., 1996; Wang, et al., 1992) and human liver (Baumgart, et al., 1996). The ACOX reaction is coupled to a reaction catalyzed by CAT; ACOX transfers electrons to FAD to form $FADH_2$, which in turn is autoxidized by oxygen to generate H_2O_2 and FAD. CAT metabolizes the H_2O_2 produced to water and molecular oxygen. Because the step catalyzed by ACOX controls the flux of carbon through β-oxidation, ACOX is ideally suited to a study of the regulation of TAG breakdown in oil seeds. ACOX has been shown to be regulated at translational level in pumpkin (Hayashi, et al., 1998). Transcription of the ACOX gene is controlled by carbon catabolite repression in *Candida maltosa* (Masuda, et al., 1995) and *Saccharomyces cerevisiae* (Luo, et al., 1996; Stanway, et al., 1995; Wang, et al., 1992). Graham, et al. (1994), have also shown that the expression of MS and ICL genes in cucumber is regulated by carbon catabolite repression.

1.10 Effect of embryo on reserve mobilization

As seen in section 1.8, storage reserve breakdown products are used for seedling establishment. There is direct correlation between the breakdown of storage reserves, the increase in activity of enzymes responsible for storage reserve breakdown, and the growth of the seedling. Consequently, the regulatory role of the embryo in storage reserve breakdown has been investigated in many species. It has been shown that the removal of embryonic axis before imbibition impairs the normal breakdown of storage reserves in cucumber (Davies and Chapman, 1979a; Davies and Chapman 1979b; Slack, et al., 1977), bean (Mirohashi, 1982; Kern and Chrispeels, 1978; Minamikawa, 1978) and pea (Chin, et al., 1972). Gifford, et al. (1984) showed that the embryonic axis is required for both rapid protein breakdown and the attainment of high levels of activity of proteolytic enzymes in castor bean endosperm. Nyman, (1971) and Ching, (1970) have shown that embryo attachment is necessary for normal reserve mobilization in gymnosperm megagametophytes. In pine, Murray and Adams (1980) and Bilderback (1974) showed that the level of ICL activity in the megagametophyte was controlled by the embryo.

It has been shown that the intracellular concentration of hexose sugars or the flux of hexose sugars into glycolysis may affect changes in expression of genes involved in glyoxylate cycle (Ismail, et al., 1997; Graham, et al., 1994). Since the products of reserve breakdown do not accumulate in the storage tissues of loblolly pine following germination, but instead they are rapidly exported to

the growing seedling, the seedling may be acting as an efficient sink for the products of reserve breakdown (Murry and Adams, 1980; Bilderback, 1974). The seedling would therefore act as an important controlling factor in reserve mobilization. High levels of storage reserve breakdown products such as carbohydrates and amino acids inhibit synthesis or activity of enzymes involved in reserve breakdown (Bewley and Black, 1994; Graham, et al., 1994, Thomas and Rodriguez, 1994; Dunaevsky and Belozersky, 1993; Davies and Chapman, 1980). It has also been suggested that osmotic regulation may alter the expression of reserve mobilizing enzymes due to metabolite accumulation. The seedling may also secrete signal molecules, like hormones, which may regulate reserve breakdown, for example, Gifford, et al., (1984) showed that gibberellins affected the activity of enzymes that are involved in protein breakdown in the endosperm of castor bean whereas Rodrigurez, et al., (1987) have reported that gibberellins act as transcriptional enhancers of genes being expressed in germinating castor bean seeds. In cereals, Yu et al. (1996) showed that expression of α-amylase genes in aleurone cells was regulated by gibberellins produced in the embryo.

1.11 The present study

The major pressures that face the forestry industry are: 1) inability of forest products to meet the current world demands, in relation to increasing demands from countries with emerging economies, particularly in Asia, and 2)

increasing pressures to reduce harvesting from native forests. Therefore, increased germinability and improved wood quality would make a significant impact on ameliorating this supply-demand problem. However, in order to make biotechnological improvements on these forest trees, it is necessary to understand the biochemistry and molecular biology of the trees first. This is why I have embarked on increasing the knowledge of the biochemical processes that occur during germination and post germinative growth in loblolly pine. In this study, I have focused on the regulation TAG catabolism, as TAGs are the major storage reserves in loblolly pine seeds. My hypothesis was that the embryo regulates megagametophyte TAG breakdown at the level of mRNA. This hypothesis was tested by studying the regulation of important enzymes in the TAG breakdown pathway in megagametophytes, in the presence and absence of the embryo.

While several glyoxysomal enzymes have been characterized in conifers (Mullen and Gifford, 1997; Mullen and Gifford, 1995a; 1995b, Mullen and Gifford, 1993; Vani, et al., 1990), ACOX has not been studied. To begin to study the regulation of this enzyme in conifers, I purified ACOX from loblolly pine to apparent homogeneity and generated antibodies to this protein. These antibodies were then used to isolate a cDNA clone for ACOX. This research was designed to provide molecular tools to enable us study the regulation of this enzyme. The effect of embryo removal on the activity, protein and mRNA levels of ACOX is presented in this thesis and is discussed in relation to the regulation of TAG breakdown during germination and post germinative growth. The effect of embryo removal on CAT activity is also presented. The data presented in this

thesis is a step towards alleviating a major problem of many economically important conifer species, which is low seed germination rates and poor seedling vigor.

2. Materials ad methods

2.1. Seed Material and Germination

Loblolly pine (Pinus taeda L.) seeds, kindly provided by Westvaco, (Summerville, SC, USA) were collected in the fall of 1992 from open pollinated first generation trees Westvaco clone (11-9). Seeds were first surface sterilized with tween 20 (Bio-Rad) and 1% NaOCl according to Groome et al. (1991) and then placed on sterile moist Kimpak (Seedboro Equipment, Chicago, IL, USA) at 2^0C (DAI_2) for 35 days in the dark to break dormancy (stratification; Schneider and Gifford 1994). Stratified seeds were surface sterilized with 1% NaOCl (Groome et al., 1991), and transferred to autoclaved Kimpak-lined germination trays. The trays were put in a germinator at 30^0C (DAI_{30}) in continuous illumination (19 mol μm^{-2} S^{-1}). Seed germination and subsequent seedling growth was staged according to radicle size classes as described in Mullen et al. (1996). Megagametophytes and seedlings were collected up to 12 DAI_{30}. For experiments that required mature desiccated seeds (non-stratified), seeds were

moistened overnight on Kimpak, surface sterilized with 1% NaOCl, decoated under sterile conditions and incubated on sterile agar plates. The seed parts that were harvested at the different time periods were frozen in liquid nitrogen and then stored at –80 ^{0}C.

2.2. Chemicals and Equipment

Research grade chemicals were purchased from Fisher Scientific (Edmonton, AB, Canada), Sigma Chemical Co. (St. Louis, MO, USA), Amersham (Oakville, ON, Canada), Bio-Rad Laboratories Ltd. (Mississauga, ON, Canada) and BDH (Toronto, ON, Canada). Radioisotopes, which included [^{35}S]-methionine, α[^{32}P]-dCTP and [^{14}C]-methylated molecular mass protein standards were purchased from Amersham. Aqueous counting scintillant (ACS-II) was obtained from Amersham. ENHANCE was purchased from Dupont (Markham, ON, Canada). The LS 6000 TA scintillation counter, the J2-21/E centrifuge, L8-60 M ultracentrifuge and rotors, microfuge E, Scintillation vials and DU-65 spectrophotometer used in this research were manufactured by Beckman (Richmond, BC, Canada). Deionized water was obtained from a Milli-Q filtration water system (Millipore Corporation, Bedford, MA, USA). Sequencing primers were purchased from Pharmacia (Montreal, QB, Canada). Restriction endonucleases were obtained from Pharmacia, GibCO BRL (Gaithersberg, MD, USA) or from Promega-Biotech (Madison, WI, USA). Agarose was supplied by

Sigma. Media reagents were purchased from DifCO Laboratories (Detroit, MI, USA). The MLB-06 miniature horizontal Gel System was purchased from Tyler Research Instruments (Edmonton, AB, Canada). Protein chromatographic columns were obtained from Bio-Rad, while the RediFrac fraction collector, was supplied by Pharmacia LKB Biotech. Mini-Protean II electrophoresis apparatus and mini-Protean II 2-D cell system for one and two dimensional gel electrophoresis respectively were purchased from Bio-Rad Laboratories Ltd (Mississauga, ON, Canada). DNA and RNA isolations were performed using kits that were purchased from QIAGEN Inc. (Mississauga, ON, Canada).

2.3. Protein and Enzyme Assays

All assays were carried out at room temperature (23^0C). The data points for enzyme activity are a mean of three independent determinations.

2.3.1. Protein Assay

Protein quantification was done by the method of Lowry et al., (1951) using bovine serum albumin as the standard. The protein content of fractions from chromatographic columns was followed spectrophotometrically at 280 nm.

2.3.2. Catalase Assay

The activity of CAT (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) in cell free extracts was measured using the method of Luck (1963). The assay was carried out in a reaction mixture containing 135 mM KH_2PO_4, (pH 7.0), 0.0125% (v/v) Triton X-100, 0.096% (v/v) H_2O_2 and the enzyme extract in a final volume of 3 ml. The control reactions had H_2O_2 omitted. The change in absorbance, as a measure of first order decomposition of H_2O_2 was monitored spectrophotometrically at 240 nm. One unit of CAT activity in kats was defined as the amount of enzyme that converted one mole of H_2O_2 to water and molecular oxygen per second at 23^0C (Extinction coefficient: $40x10^3$ M^{-1} cm^{-1}; Aebi, 1974).

2.3.3. Acyl-CoA Oxidase Assay

Acyl-CoA oxidase (EC 1.3.3.6) activity was measured by following the first-order rate of H_2O_2 formation, according to Alain et al (1974) with modifications. The final assay reaction mixture contained 45 mM MES, 0.04% (w/v) palmitoyl-CoA, 0.70 mM 4-aminoantipyrine, 0.04 mM flavin adenine dinucliotide, 9.60 mM phenol, 0.09% (v/v) Triton X-100, 15 purpurogallin units peroxidase and 0.2 ml of the enzyme extract in a total volume 1.4 ml. ACOX uses palmitoyl-CoA as a substrate, to produce H_2O_2. The H_2O_2 produced reacts with 4-aminoantipyrine and phenol in a peroxidase coupled reaction to produce a quinoneimine dye that was measured spectrophotometrically at 500 nm. One unit of ACOX activity in kats was defined as the conversion of one mole of palmytoyl-CoA to H_2O_2 and

hexadecenoyl-CoA per second at pH 8.0 at 23^{0}C (extinction coefficient of quinoneimine dye at 500 nm: 12.78 $M^{-1}cm^{-1}$).

2.4 Purification of ACOX

All operations were carried out at 4^{0}C unless stated otherwise. The procedure of Kirsch et al., (1986) was followed with modifications. About 40 g of isolated loblolly pine megagametophytes, collected 9-11 DAI_{30}, were ground in liquid nitrogen using a cold mortar and pestle, and the powder extracted in 100 ml cold acetone (-20^{0}C). The homogenate was centrifuged at 25 000 × g for 30 min and the pellet resuspended in 80 ml Tris buffer which contained 150 mM Tris-HCl pH 7.5, 10 mM KCl, 1 mM EDTA, 25% (w/v) sucrose. The suspension was homogenized in a Brinkman Polytron blender at high speed and then filtered through a cheesecloth that had been soaked in the Tris buffer. The filtrate was subjected to centrifugation at 25 000 × g for 30 min. The supernatant was heated in a water bath at 55^{0}C for 5 min, and then cooled to 2^{0}C in an ice bath. The precipitated material, was removed by centrifugation at 100 000 × g for 60 min. The supernatant was brought to 45% saturation by adding solid ammonium sulfate. The suspension was stirred for 30 min and the precipitate was collected by centrifugation at 12 000 × g for 20 min. The pellet was dissolved in 20 ml of 20 mM sodium phosphate buffer (pH 7.5) containing 10% sucrose, and the solution was applied to a 1.5 x 25 cm column containing phenyl-Sepharose CL-

4B (Sigma) equilibrated with 20 mM sodium phosphate buffer, (pH 7.5), that contained 10% sucrose. After extensive washing with 40% ethylene glycol in 12 mM sodium phosphate buffer (pH 7.5) that contained 10% sucrose, the column was eluted with a continuous gradient of 40 to 60% ethylene glycol in the 12 mM phosphate buffer. The peak ACOX activity fractions obtained following elution were pooled and applied to a hydroxylapatite Bio-Gel HTP column (1×12 cm; Bio-Rad) equilibrated with 20 mM sodium phosphate buffer (pH 7.5), that contained 10% sucrose. Following extensive washing with the phosphate-sucrose buffer, protein was eluted with a continuous 20-120 mM gradient of sodium phosphate buffer that contained 10% sucrose. The peak ACOX activity fractions were pooled and concentrated using centricon-10 microconcentrators according to manufacturers' instructions (Amicon). The resulting 3 ml were then chromatographed on a 1.5×100 cm Ultrogel AcA-34 column equilibrated with 20 mM phosphate buffer, (pH 7.5) that contained 10% sucrose. Pure ACOX fractions were collected, concentrated as before and stored at -70^{0}C.

2.5 Molecular mass determination

The native molecular mass of ACOX was determined using a 1.5 × 100 cm Utrogel AcA-34 column that was equilibrated with 20 mM phosphate buffer, pH 7.5 containing 10% sucrose. Blue dextran 2000 (Pharmacia) was used to determine the void volume of the column. Molecular weight markers that were

used, were: throglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa) (Sigma). Proteins were eluted, using the equilibration buffer at a flow rate of 10 ml per hr.

2.6. ACOX Antibody Production

Polyclonal antibodies were prepared using 30 µg of the purified ACOX protein in 0.75 ml PBS mixed with 0.75 ml of Freund's complete adjuvant. This was injected subcutaneously and inter-scapularly at multiple sites in a male Flemish giant × French lop rabbit. Two separate booster injections of 30 µg and 15 µg respectively were given every after 28 days according to the initial injection procedure except that the antigen was mixed with Freund's incomplete adjuvant. Ten days following booster injections, rabbits were bled to test for titer. Blood samples were taken via ear bleeding, incubated at 37^0C for 2 h and then at 4^0C overnight. The precipitate was removed by centrifugation at maximum speed in a clinical centrifuge and the serum stored at -80^0C.

2.7 *In vivo* protein labeling

Ten megagametophytes were incubated with 100 µCi ($3.7x10^6$ Bq) of [^{35} S]-methionine (specific activity of 1.5-18.5 GBq/mmol) in 100 µl at 30^0C for 3hrs (Groome et al., 1991). The megagametophytes were then rinsed with water to remove excess label and ground using a mortar and pestle. The slurry was centrifuged at 10 000 x g for 15 min and the supernatant was collected. The percent incorporation into protein was determined by TCA precipitation using the method of Gifford and Bewely (1984).

2.8 Electrophoresis

2.8.1 SDS-PAGE

Single-Dimension SDS-PAGE was carried out in 0.75 mm 12% acylamide slab gels on a mini Protean II electrophoresis apparatus (Bio Rad) according to Laemmli (1970). Relative molecular weights of proteins were determined by the method of Weber and Osborne (1969). Molecular weight standards included lysozyme, 14.4 kDa; soybean trypsin, 21.5 kDa; bovine carbonic anhydrase, 31 kDa; hen egg white ovalbumin, 45 kDa; bovine serum albumin, 66.2 kDa; rabbit muscle phosphorylase b, 97.4 kDa (Bio Rad). Proteins were either visualized following staining with coomassie brilliant blue R or by silver staining using a Bio-Rad silver stain kit according to the manufacturer's instructions. For

fluorography, gels were first gently shaken in a solution of 60% methanol and 10% acetic acid for 30 min to fix the proteins, and then treated with EN^{3}HANCE (Dupont) for 2 hrs according to the manufactures' instructions. The gels were dried in a Bio-Rad Model 543 Gel Dryer and then exposed to X-OMAT AR film (Kodak) at –80^{0}C. [^{14}C]-methylated molecular mass markers used were: myosin, 200 kDa; phosphorylase b, 92.5 kDa; BSA, 69.0 kDa; ovalbumin, 46.0 kDa; carbonic anhydrase, 30.0 kDa; and lysozyme, 14.3 kDa (Amersham).

2.8.2 2D-SDS-PAGE

Two-dimensional gel electrophoresis (IEF/SDS-PAGE) was carried out with a mini-Protean II 2-D cell system (Bio-Rad) as follows: protein containing 9.5 M urea were loaded at the cathode end of tube gels that contained 9.2 M urea, 4% (v/v) polyacrylamide (30%T/5.4%C), 1.6% (v/v) pharmalyte (pH 5-8) and 4% pharmalyte (pH 3-10) (Sigma). The cathode and anode solutions (20 mM NaOH and 10 mM H_3PO_4 respectively) were degassed thoroughly overnight before use. For the first dimension, IEF gel electrophoresis, the tube gels were pre-electrophoresed at 200 V for 10 min, 300 V for 15 min and 400 V for 15 min. IEF was then carried out at 500 V for 10 min followed by 750 V for 3.5 hrs. The second dimension was performed using a 1 mm 12% gel as described in section 2.7.1.

2.8.3 Isoelectric focusing

Isoelectric focusing was carried out on a mini Protean II electrophoresis apparatus (BioRad). Protein samples containing 9.5 M urea were loaded at the cathode of a tube gel that contained 9.2 M urea, 4 % (v/v) polyacrylamide, 1.6 % (v/v) pharmalyte (pH 5-8) and 0.4 % (v/v) pharmalyte (pH 3-10). The cathode (upper) and anode (lower) gel buffer solutions were 20 mM NaOH and 10 mM H_3PO_4, respectively; they were degassed thoroughly before use. A mixture of protein standards (Broad range PI 4.6-9.6; Bio Rad) were run in parallel to the samples. Gels were silver stained (Bio Rad). The migration of the sample bands and standards were measured and the pI was determined by interpolation.

2.9 Western Transfer

After SDS-PAGE, gels were equilibrated for 17 minutes in transfer buffer (25 mM Tris, 192 mM glycine and 20% v/v methanol), containing 0.015% SDS. The transfer of proteins to nitrocellulose (Bio-Rad) was carried out overnight at 4^0C at a constant voltage of 30 V in a mini Protean II Trans-Blot Cell apparatus (Bio-Rad). The efficiency of protein transfer to the nitrocellulose membrane was measured by Ponceau S staining as described in Sambrook et al. (1989). The subsequent immunoblotting was done using the method of Kunce and Trelease (1986). Blots were blocked for 1 hr at room temperature in "blotto", which is 3% (w/v) non-fat dry milk (Carnation), 0.15 M NaCl and 20 mM Tris-HCl (pH 7.8).

The blots were incubated with primary antibody (diluted 1:5000) for 2 hrs at room temperature and then washed 3 times in the "blotto" solution. The secondary alkaline phosphatase-goat-anti-rabbit antibody (diluted 1:3000;Sigma) was added and incubated for 2 hrs at room temperature in "blotto" solution. After three 20-minute washes, cross-reactive proteins were visualized using alkaline phosphatase activity and NBT/BCIP according to the manufacturer's instructions (Bio-Rad).

2.10 Immunotitration

For immunotitration experiments, the method of Trelease et al. (1987) was used with modifications. One ml portions of megagametophyte extracts prepared in MES buffer were incubated with 0, 25, 50, 75 and 100 µl/ml ACOX antibody or preimmune serum for 1 hr at room temperature followed by overnight incubation at 4^0C in 1.5 ml microfuge tubes. The tubes were centrifuged at 12 000 x g for 15 min in a microfuge (Beckman) and the supernatants were assayed for ACOX activity.

2.11 Immunoprecipitation

Ten megagametophytes for each stage were labeled with 100 µCi ($3.7x10^6$ Bq) [^{35}S]-methionine for 3 hrs as described previously. *In vivo*

immunoprecipitation of the [^{35}S]-methionine labeled protein was carried out using the combined methods of Harlow and Lane (1988) and Sambrook et al (1989). Following TCA precipitation (Gifford and Bewley, 1984) to determine the amount of incorporation of [^{35}S]-methionine, 10 µl of the labeled protein solution (containing approximately 300 000 dpm) was brought up to 1 ml with buffer that contains 1% Triton X-100 (v/v), 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 20 mM methionine, 3 mM EDTA, and 2µg/ml aprotinin. This mixture was incubated on ice at 4°C for 15 minutes and then centrifuged for 15 minutes at 10 000 x g at 4^{0}C. Solid NaCl was added to the supernatant to make it 1 M. This mixture was precleared by incubating it with preimmune serum for 1 hr on ice and then incubated for another 30 min on ice with 50 µl of 10% *Staphylococcus aureus* Cowan I cells (Sigma Chemical Co.) which had been washed 3 times in the above Triton X-100-Tris buffer. The mixture was centrifuged for 15 minutes at 10 000 rpm at 4°C. To the supernatant, 25 µl of ACOX antibody was added and incubated for 1 hr at room temperature and then overnight on a nutator at 4^{0}C. Immune complexes were collected by addition of 50 µl of the *Staphylococcus aureus* Cowan I cells that had been pre-washed 3 times in the Triton X-100-Tris buffer. After 1 hr incubation at room temperature with constant mixing on a nutator, cells were collected by centrifugation at 10 000 x g for 15 minutes at 4^{0}C. The cells were washed 5 times in buffer similar to the one described above except that 0.1% Triton X-100 was used instead of 1%. The cells were further washed 3 times in the above buffer that has Triton X-100 omitted altogether. Immune complexes were dissociated by boiling in Laemmli buffer (1:1 ratio) at 95^{0}C for 5

minutes (Laemmli, 1970). This was followed by centrifugation at 10 000 x g for 15 minutes at room temperature. An aliquot of the resulting supernatant was analyzed by SDS-PAGE and visualized using fluorography. The remainder was stored at -80^{0}C.

2.12 Immunoscreening of cDNA Library with ACOX Antibody

Approximately 2.5x10^5 plaques (5x10^4 pfu per plate) were transferred from a loblolly pine λ cDNA megagametophyte library (Mullen 1995) to nitrocellulose membranes (nitroplus, MSI, Westboro, MA, USA) and probed with ACOX antiserum using a picoBlue immunoscreening kit (Stratagene) with modifications. Bacterial cells (XL1-Blue MFR') were infected with the phage from the library. The infected cells were mixed with top agar and plated, and the plates were incubated at 42^{0}C for ~3.5 hrs. The plates were then overlayed with nitrocellulose membranes that had been presoaked in 10 mM IPTG for 30 min and air dried, and then incubated for a further 3.5 hrs at 37^{0}C, and overnight at 4^{0}C. Membranes were pierced in 3 non-symmetrical positions for the purpose of identifying positive plaques. Immunodetection was carried out using ACOX antibody (diluted 1:2000) and goat anti-rabbit conjugated alkaline phosphatase with NBT/BCIP as substrates, according to supplers' instructions (Stratagene). Positive plaques were further purified by 3 successive screens. *In vivo* excision with ExAssist helper phage and SOLR *E.coli* cells was used to rescue cDNA

inserts from the UNI-ZAP XR vector to form pBluescript (SK-) phagemids. The excised pBluescript (SK-) phagemids were selected on the basis of ampicillin resistance. Phagemids were plated for single cell colonies on plates containing 50 µg/ml ampicillin. Plasmid DNA was isolated using QiAprep Spin Miniprep Kit (QIAGEN) according to the manufacture's instructions. The cDNA inserts were excised by ECOR 1/Kpn 1 restriction digests and then analyzed by Agarose gel electrophoresis.

2.13. DNA sequencing

DNA sequencing was carried out by the Arizona State University DNA sequencing facility (Tempe Arizona), using IBI Prism 377 Sequencer (Perkin Elmer Biosystems, Foster City, CA, USA). Sequencing was from 3' and 5' ends of the cDNA inserts with T7 and M13 oligonucleotide reverse primers. To get a full sequence, successive rounds of sequencing were done using custom designed oligonucleotide primers that were made at the same facility. BLAST Server (NCBI, Bethesda, MD, USA) and DNAman software (Lynnom Biosoft, Vandreuil, Quebec, Canada) were used for DNA and amino acid sequence analysis.

2.14 RNA Isolation and Northern Blot Analysis.

Total RNA for northern blot analysis was isolated from 100 mg of frozen megagametophytes using Rneasy Plant Mini Kit ((QIAGEN) according to the supplier's instructions. RNA quantification was carried out spectrophotometrically and RNA integrity was determined by ethidium bromide staining of the agarose gel. Northern blot analysis was performed using 10 µg of total RNA, which was denatured with formaldehyde at 65^{0}C and then separated on a 1.2% agarose formaldehyde/formamide gel according to Sambrook et al. (1989). After electrophoresis, the gels were washed twice in 10x SSC buffer for 20 min each. The RNA was passively transferred onto Zeta probe blotting membrane (Bio-Rad) in 10x SSC. The membranes were washed twice with 2x SSC, air-dried and RNA was fixed to membranes by baking at 80^{0}C for 30 min under vacuum. The membranes were pre-hybridized in 0.5 M Na_2HPO_4 (pH 7.2), 7% SDS, 1 mM EDTA for 1 hr at 65^{0}C. Hybridization with a 1.5 kb ACOX ^{32}P-labelled probe, which was made using a T7 oligolabelling Kit (Pharmacia Biotech) according to the manufacture's instructions, was performed overnight under the same conditions as pre-hybridization conditions. The blots were washed twice in 50 mM Na_2HPO_4 (pH 7.2), 5% SDS, 1 mM EDTA for 20 min at 65^{0}C. This was followed by a more stringent wash with 50 mM Na_2HPO_4 (pH 7.2), 1% SDS, 1 mM EDTA for 2 x 20 min at 65^{0}C. The blot was wrapped in saran wrap and exposed to Kodak X-OMAT film at -80^{0}C using intensifying screens.

2.15. Determination of Triacylglycerols

Triacylglycerols were determined using the method of Feirer et al. (1989), with modifications. Tissue samples (20-200 mg fresh weight) were homogenized with 1.25 mL isopropanol. The homogenate was shaken for 15 min on a nutator, then centrifuged at 14,000 rpm for 15 min, and the supernatant was collected. The pellet was re-suspended and re-extracted once in 1.25 mL isopropanol, and the supernatants were pooled. Of the pooled crude supernatant, 800 µL were added to test tubes containing 0.8 g alumina and 1.8 mL isopropanol. Tubes were capped and gently shaken for 15 min on a nutator, centrifuged in a clinical centrifuge at 10 000 x g for 20 min and the supernatant was collected. The alumina pellet was washed twice with 2.6 mL isopropanol and each time centrifuged at 10 000 x g for 20 min. and the supernatants were collected; all supernatants were pooled. TAG quantification was performed using the following reaction mixture: 800 µl of the pooled supernatant and 200 µl of 1N KOH were added to a polypropylene test tube and mixed well before being incubated in a 60 ^{0}C water bath for 5 min. The solution was cooled to room temperature after which 200 µl of sodium periodate (125 mg $NaIO_4$ in 50 ml acetic acid) was added to each tube. After 10 min, 1.2 ml of color reagent (40 ml 2M ammonium acetate, 150 µl acetylacetone, and 80 ml isopropanol) was added to each tube and the tubes were returned to the 60^{0}C water bath for 30 min. The absorbance of the color developed was measured spectrophotometrically at 410 nm. Standards, using triolein, (which provided a linear standard curve that suited

the TAG levels in the tissues used for the experiment) were carried out throughout the experiment. A linear regression based on the standards which gave a good correlation coefficient (>99%) was used to convert the unknown absorbance to µg/ml TAGs.

2.16 Carbohydrate Determination

Soluble and insoluble carbohydrates were extracted using a modification of the method of Joy et al. (1991). Forty to 200 mg (fresh weight) of tissue were homogenized with 1 mL 80% (v/v) ethanol and gently mixed for 1 h on a nutator. The mixture was then centrifuged at 12 000 x g for 15 min at room temperature, and the supernatant was collected. The pellet was re-extracted twice with 1 mL 80% ethanol and 15 min incubations. Pooled supernatants contained the 80% ethanol-soluble carbohydrates. The insoluble carbohydrates were extracted by re-suspending the above pellet in 1 mL cold Milli Q H_2O and then adding 1.5 mL 52% perchloric acid. The mixture was incubated on ice for 20 min. After this incubation, a further 2.5 mL cold Milli Q H_2O was added to the mixture. The mixture was centrifuged at 12 000 x g for 30 min at 4°C. The supernatant was collected by filtering through a Whatman #1 filter in a funnel. The pellet was re-extracted once by the same procedure and the supernatants were pooled. Soluble and insoluble carbohydrates were quantified using the method of Joy et al. (1991). A mixture of 0.5 mL sample and 0.5 mL of 5% phenol solution was incubated for

30 min, 3.0 mL concentrated H_2SO_4 was then added and mixed immediately by vortexing. The absorbance was measured spectrophotometrically at 490 nm. Anhydrous D-glucose (BDH) was used as a standard.

3 RESULTS

3.1 ACOX activity in megagametophytes

The optimum pH for ACOX activity in cell-free extracts of megagametophytes was determined to be 8, therefore subsequent ACOX activity determinations were carried out at this pH. The results depicted in Figure 3.1 indicate that ACOX activity in cell-free was undetectable in megagametophytes from mature seeds, and extremely low in megagametophyte from stratified seeds. At 3 DAI_{30} there was measurable ACOX activity, after which ACOX activity increased steadily reaching a maximum between 9-11 DAI_{30}; ACOX activity then declined.

3.2 ACOX enzyme purification

Maximum ACOX activity occurred in the megagametophyte 9-11 DAI_{30} (Figure 3.1), since this time period coincided with maximum rates of lipid degradation in this tissue (Stone and Gifford 1997), ACOX purification was performed using megagametophytes collected 9-11 DAI_{30}.

The six purification steps used are summarized in Table 1 and Figure 3.5, lanes, A-F. Following ammonium sulphate precipitation, chromatography on Phenyl-Sepharose (Figure 3.2) separated ACOX from most other proteins. Hydroxyapatite chromatography (Figure 3.3), gave a significant enrichment of ACOX specific activity (Table 1). Finally, molecular sieving on Ultrogel AcA-34 (Figure 3.4) resulted in a homogeneous enzyme with specific activity of 417.5 nkats per mg protein (Table 1); purification was 1250 fold resulting in a 9% yield. This protein resolved into a single 71 kDa band on SDS-PAGE under reducing conditions (Figure 3.5, lane F).

Antibodies to the purified protein were prepared in rabbits. Western blot analysis of a crude extract using these antibodies showed a single 71 kDa band (Figure 3.5, lane G).

Using a molecular sieving chromatographic column, the molecular mass of the holo-enzyme was shown to 150 kDa (Figure 3.6).

Figure 3.1 Changes in ACOX activity in megagametophytes following seed imbibition at 30^0C. Each data point is a mean of three independent determinations.

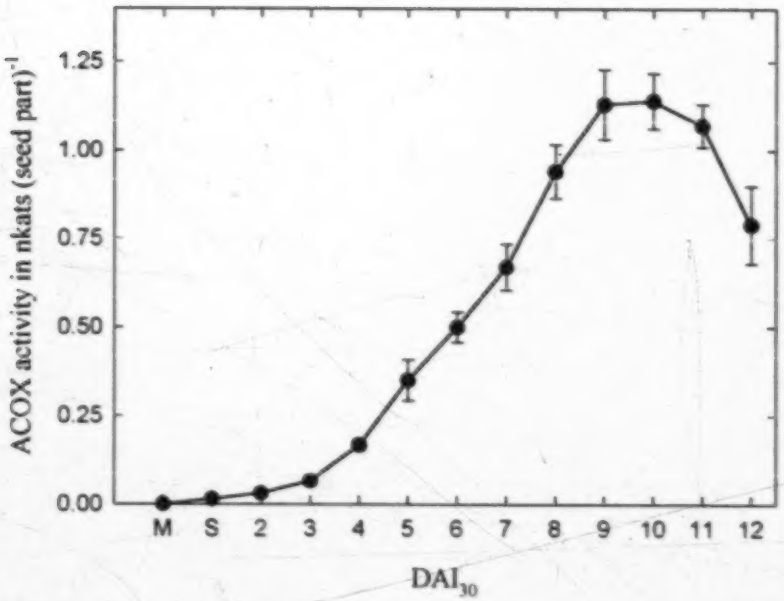

ACOX activity in nkats (seed part)$^{-1}$
1.25
1.00
0.75
0.50
0.25
0.00
M S 2 3 4 5 6 7 8 9 10 11 12
DAI$_{30}$

Figure 3.2. Fractionation of proteins by hydrophobic chromatography. Following ammonium sulphate precipitation, the protein solution was applied to a phenyl sepharose column and polar proteins were eliminated from the column by extensive washing with 40% ethylene glycol. This was followed by applying increasing concentrations of ethylene glycol on the column. Protein A_{280} (open circles) and ACOX activity (closed circles). A straight line indicate ethylene glycol concentrations.

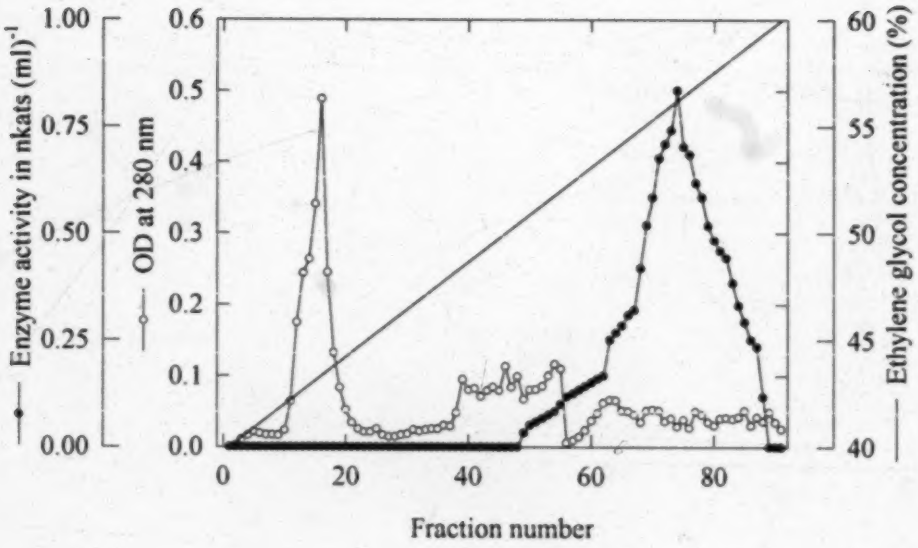

Enzyme activity in nkats (ml)-1
OD at 280 nm
Ethylene glycol concentration (%)
Fraction number
1.00
0.75
0.50
0.25
0.00
0.6
0.5
0.4
0.3
0.2
0.1
0.0
60
55
50
45
40
0
20
40
60
80

Figure 3.3. Separation of acyl-coA oxidase on hydroxylapatite column. Peak acyl-coA activity fractions from phenyl sepharose column were loaded onto hydroxylapatite column equilibrated with 20 mM phosphate buffer, pH 7.5. The bound protein was eluted by a linear phosphate gradient ranging from 20-120 mM. Protein A_{280} nm (open circles) and ACOX activity (closed circles). A straight line indicate phosphate concentrations.

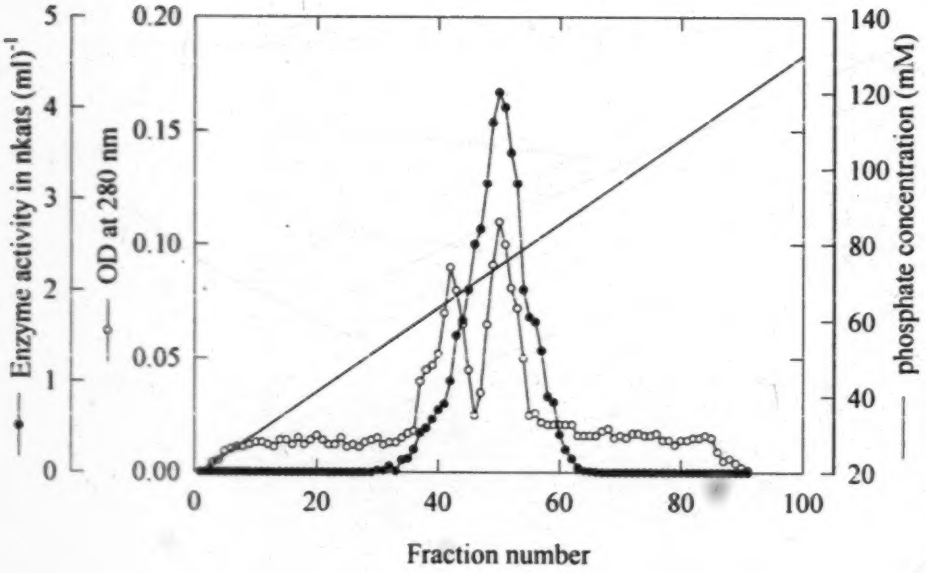

Enzyme activity in nkats (ml)-1
OD at 280 nm
phosphate concentration (mM)
Fraction number
0
1
2
3
4
5
0.00
0.05
0.10
0.15
0.20
20
40
60
80
100
120
140
0
20
40
60
80
100

Figure 3.4. Purification of Acyl-coA oxidase on Ultrogel AcA-34. Peak ACOX activity fractions from hydroxylapatite column were concentrated to 3 ml and then loaded onto Ultrogel AcA-34 column equilibrated with 20 mM phosphate buffer, pH 7.5. The column was then washed by a continuous flow (12.5 ml/hr) of the phosphate buffer and a sharp peak of enzyme activity that gave a homogeneous enzyme was achieved

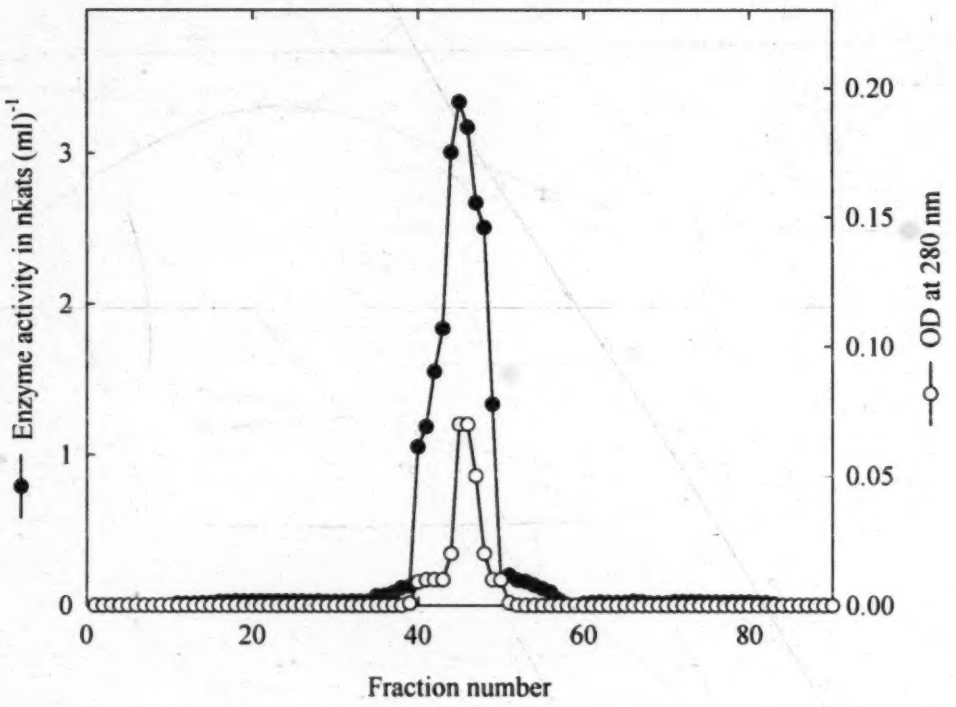

Enzyme activity in nkats (ml)-1
OD at 280 nm
Fraction number
0
1
2
3
0.00
0.05
0.10
0.15
0.20
0
20
40
60
80

Figure 3.5. Progress of purification of loblolly pine acyl-coA oxidase as shown by gel electrophoretic analysis. Fractions were pooled from each stage of the purification procedure (Table 1), were subjected to SDS-PAGE on 12% gels and visualized either by coomassie blue staining (A-E) or silver staining lane F. *Lane* A, crude extract; *lane* B, heat treatment; *lane* C, 45% saturation $(NH_4)_2SO_4$ fraction; *lane* D, pooled fractions from phenyl sepharose column; *lane* E, preparation following chromatography on hydroxylapatite column; *lane* F, purified enzyme as obtained following the molecular sieving column. *Lane* G shows a western blot analysis of megagametophyte cell free extract probed with loblolly pine anti-ACOX serum.

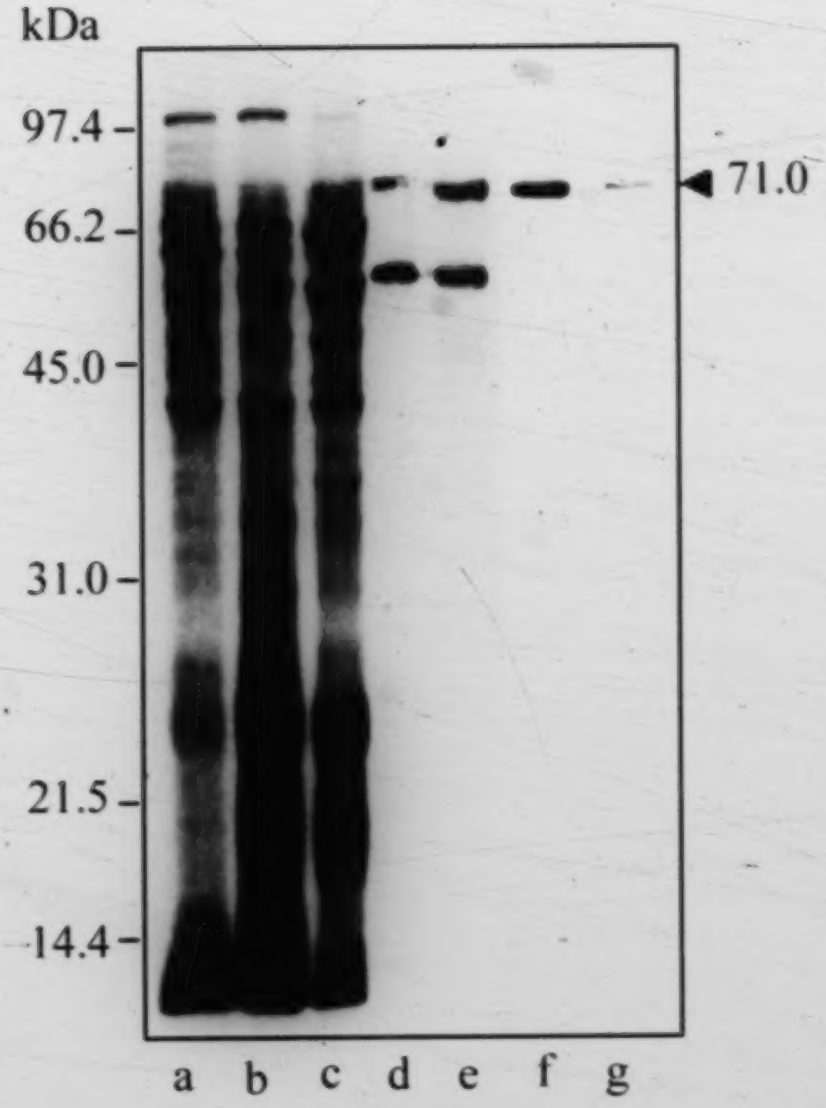

kDa
97.4
66.2
45.0
31.0
21.5
14.4
71.0
a
b
c
d
e
f
g

TABLE I. Purification procedure of acyl-CoA oxidase from 40 g of 9-11 DAI_{30} loblolly pine megagametophytes.

Purification step	Total protein (mg)	Total activity (nkats)	Yield (%)	Specific activity (nkats·mg^{-1})	Purification fold (~ fold)
Crude	1566	534.4	100	0.33	1
Heat treatment	522	400.8	75	0.84	2.5
Ammonium sulphate precipitation	34	250.5	47	7.4	22
Phenyl sepharose	2.9	150.3	28	51.8	155
Hydroxyl apatite	0.48	116.9	22	243.5	729
Ultrogel AcA-34	0.12	50.1	9	417.5	1250

3.3 Immunological characterization of ACOX

A number of immunological experiments were carried out to determine the specificity of ACOX antibodies. Immunotitration of ACOX enzyme activity (Figure 3.7), showed that 25 µl/ml of anti-ACOX serum eliminated 75% of ACOX activity in cell free extracts of 10 DAI megagametophytes. In contrast, the control using preimmune serum showed no reduction in activity of the enzyme even if 100 µl/ml of serum were used.

3.4 Tissue distribution of ACOX protein

Preliminary studies using enzyme assays showed that ACOX activity in cell-free extracts was primarily megagametophytic. The ACOX antibodies were used to further examine the tissue distribution of this enzyme. Western blot analysis (Figure 3.8) indicated that the enzyme was almost exclusively in the megagametophyte; trace amounts of the protein were detected in the shoot pole. This result is comparable to other studies of tissue localization of glyoxysomal enzymes performed on loblolly pine, which showed that the activities of CAT, ICL and MS were predominantly megagametophytic (Mullen and Gifford 1995a, 1995b).

Figure 3.6. Molecular mass determination of loblolly pine ACOX by molecular sieving on Ultrogel AcA-34. Molecular weight markers that were used in the column were: *a* cytochrome C (12.4 kDa), *b* carbonic anhydrase (29 kDa), *c* BSA (66 kDa), *d* β-amylase (200 kDa), *e* apoferritin (443 kDa, *f* thyglobulin (669 kDa). The values shown are a mean of duplicate experiments.

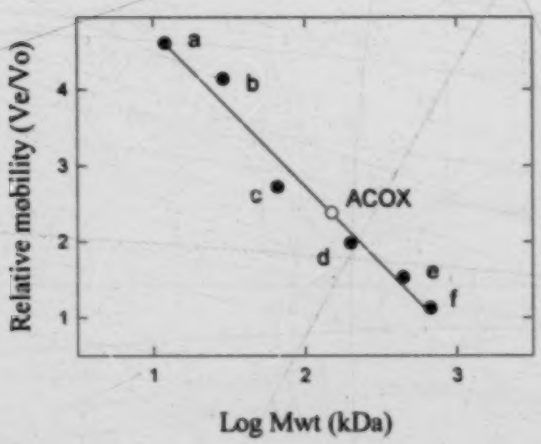

Relative mobility (Ve/Vo)
Log Mwt (kDa)
a
b
c
d
e
f
ACOX
1
2
3
4

Figure 3.7. Immunotitration of ACOX activity. Megagametophyte extracts of 10 DAI_{30} in 0.05 M $NaPO_4$ buffer, pH 7.5, were diluted to contain equivalent units of ACOX activity for each reaction. Various amounts of anti-ACOX serum or preimmune serum were added and incubated overnight. Samples were centrifuged and supernatants assayed for ACOX activity. Each value is a mean of three determinations.

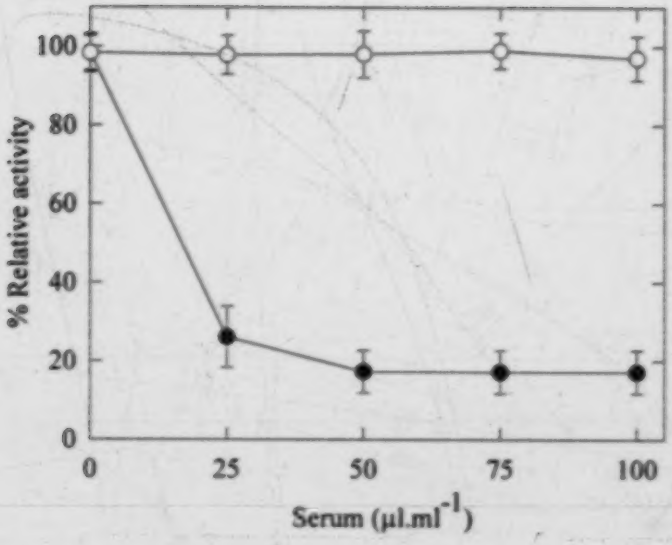
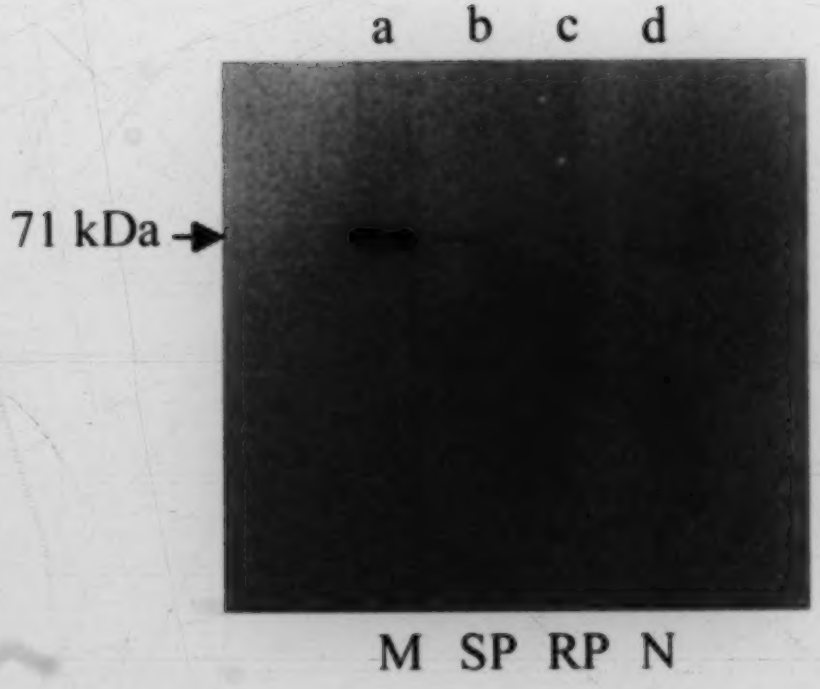

100
80
60
40
20
0
% Relative activity
0
25
50
75
100
Serum (µl.ml-1)

Figure 3.8. Tissue distribution of ACOX protein. Cell free extracts from root poles, shoot poles, needles and megagametophytes of germinated loblolly pine seeds were subjected to western blotting following SDS-PAGE. *Lane* a, megagametophyte, *lane* b, shoot pole, *lane* c, root pole, *lane* d, needle. 10 µg of protein were loaded in each lane.


a b c d
71 kDa
M SP RP N

3.5 Determination of ACOX pI

To determine the pI of ACOX, the protein was separated by IEF in the presence of 9.5 M urea. The isoelectric point of loblolly pine ACOX was 8.2, which compares to pI 7.8 for cucumber (Kirsch et al., 1986) and pI 9.2 for the mammalian liver enzyme (Osumi et al., 1980).

3.6 Isolation and characterization of an ACOX cDNA

Approximately 2.5 x 10^5 independent clones of an 8 DAI_{30} megagametophyte λ ZAP cDNA library (Mullen and Gifford 1997) were transferred to nitrocellulose filters and screened with ACOX antiserum. After four rounds of screening, five purified positive plaques were *in vivo* excised from λ into pBluescript and analyzed by EcoR I/Kpn I digestion and agarose gel electrophoresis. These clones contained cDNA inserts ranging from 0.9-1.3 kb. Sequencing of the longest clone showed that it was a partial ACOX cDNA sequence encoding the 3' end of the gene; it was designated ACOX41. Figure 3.9 shows the nucleotide and deduced amino acid sequences for ACOX41. This partial sequence was 1318 bp in length with a 153 bp 3' untranslated region, and a 1164 bp open reading frame; it generated a deduced amino acid sequence of 388 residues. The 3' untranslated region, contained an 18 bp poly $(A)^+$ tail. A peroxisomal targeting signal (the SKL motif; Rachubinski and Subramani 1995) was present at the C-terminal of the open reading frame (boxed in Figure 3.9).

The open reading frame also contained an FAD binding motif starting from amino acid 123 (double boxed in Figure 3.9).

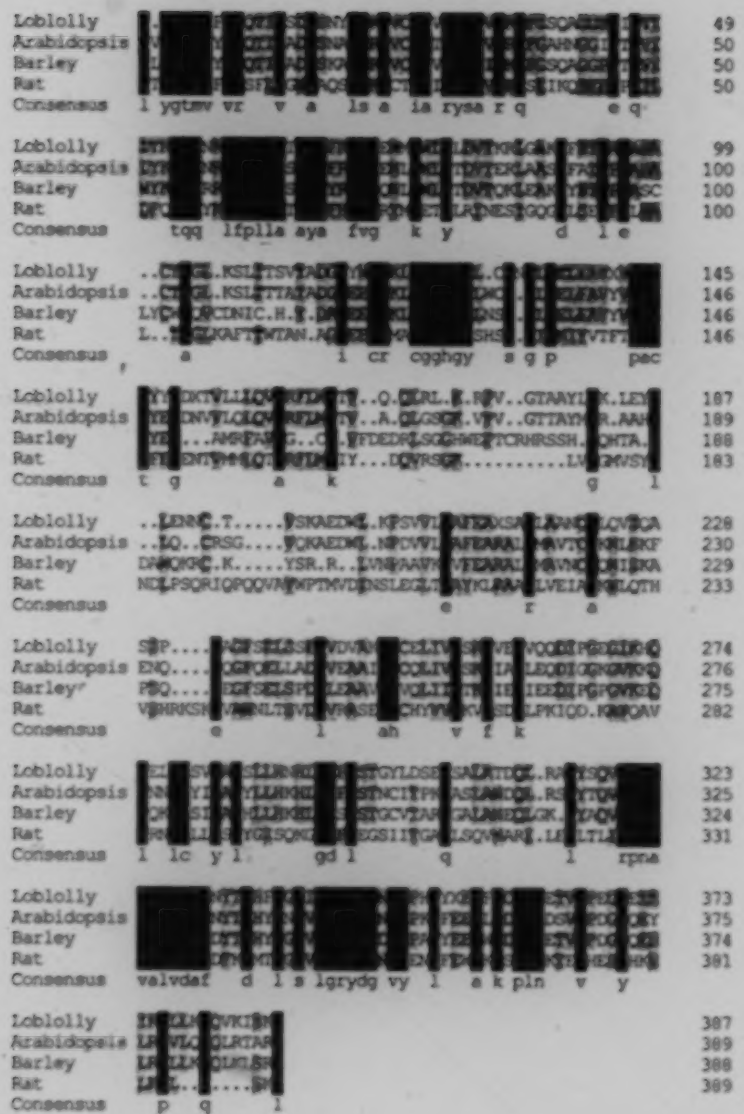
3.7 A comparison of loblolly pine ACOX deduced amino acid sequence with ACOX amino acid sequences from other species.

The deduced amino acid sequence of loblolly pine ACOX was compared with ACOX sequences from barley (Genbank accession # T04418), arabidopsis (Genbank accession # AA13498) and rat (GenBank accession # 059036). Figure 3.10 shows that there were many domains of identity shared by the four sequences; these are marked in dark blue. Light gray shading indicates regions of similarity. At 71%, Arabidopsis showed the highest level of identity to the deduced loblolly pine amino acid sequence. This was followed by barley at 68%, and rat at 42%. This sequence alignment was further analyzed to reveal that loblolly pine ACOX share many signature sequences with these species including the same targeting signal at the C-terminal (SKL and their variants) and the FAD binding motif (CGGHGY) (Dubourdieu and Fox 1977).

Figure 3.9. Nucleotide and deduced amino acid sequences of loblolly pine ACOX cDNA. The FAD binding motif (CGGHGY) is double boxed and the PTS1, C-terminal targeting sequence ser-lys-leu (SKL) is single boxed.

```
1       CTTTATGGAACCATGGTTTTTGTGCGACAAACAATTGTCTCAGATGCTTCTAATTACCTC
1        L  Y  G  T  M  V  F  V  R  Q  T  I  V  S  D  A  S  N  Y  L

61      TCCCGGGCAGTTTGCATTGCTGTGAGATACAGTGCTGTCAGACGACAATTTGGTTCACAG
21       S  R  A  V  C  I  A  V  R  Y  S  A  V  R  R  Q  F  G  S  Q

121     GCTGGTGGTCCTGAAATTCAGGTGATTGATTACAAGACTCAGCAAAACAGGCTCTTTCCC
41       A  G  G  P  E  I  Q  V  I  D  Y  K  T  Q  Q  N  R  L  F  P

181     TTGCTGGCTACAGCTTATGCCTTCCGATTCGTGGGTGAATGGATGAAATGGCTATACTTG
61       L  L  A  T  A  Y  A  F  R  F  V  G  E  W  M  K  W  L  Y  L

241     GATGTAACAAAACGTTTGGGAGCAAAGGATTTCTCAACATTGGCTGAAGCACATGCATGT
81       D  V  T  K  R  L  G  A  K  D  F  S  T  L  A  E  A  H  A  C

301     ACTGCTGGGTTAAAGTCATTGACAACATCAGTGACTGCGGATGGCATTTATAATTGTCGT
101      T  A  G  L  K  S  L  T  T  S  V  T  A  D  G  I  Y  N  C  R

361     AAGCTTTGTGGTGGACATGGGTACTTGTGCAGTAATGGGCTTCCAGAGCTGTTTGCTGTA
121      K  L  C  G  G  H  G  Y  L  C  S  N  G  L  P  E  L  F  A  V

421     NATGTTCCTGCGTGCACATATTACGGAGATNACACAGTTCTGCTTCTACAGGTAGCAAGA
141      X  V  P  A  C  T  Y  Y  G  D  X  T  V  L  L  L  Q  V  A  R

481     TTCTTGATGAAGACTGTCCAACAACTAAGATTAAAGAGGCCTGTGGGCACTGCGGCATAT
161      F  L  M  K  T  V  Q  Q  L  R  L  K  R  P  V  G  T  A  A  Y

541     TTGGGCAAGTTGGAATATCTGTTGGAGAATAACTGCACTGTTTCTAAAGCTGAAGATTGG
181      L  G  K  L  E  Y  L  L  E  N  N  C  T  V  S  K  A  E  D  W

601     CTGAAGCCTTCAGTTGTGTTGGAGGCATTTGAGGCGAGKTCAGCAAGGCTTGCTGCAAAT
201      L  K  P  S  V  V  L  E  A  F  E  A  X  S  A  R  L  A  A  N

661     TGTGCATTACAAGTCAGCCAAGCATCCAGTCCAGAAGCAGGTTTCAGTGAACTATCTAGT
221      C  A  L  Q  V  S  Q  A  S  S  P  E  A  G  F  S  E  L  S  S

721     GAGCTGGTAGACGTAGCAAAAGCTCATTGTGAGTTGATAGTTGTTTCAAAATTTGTAGAG
241      E  L  V  D  V  A  K  A  H  C  E  L  I  V  V  S  K  F  V  E

781     AAAGTACAGCAAGACATTCCAGGAGAGGGAATCAAACATCAGCTTGAGTTGTTATGCAGT
261      K  V  Q  Q  D  I  P  G  E  G  I  K  H  Q  L  E  L  L  C  S

841     GTGTATGCACTATCATTGCTGCACAACCATCTTGGTGATTTCCTTTCCACTGGTTACCTT
281      V  Y  A  L  S  L  L  H  N  H  L  G  D  F  L  S  T  G  Y  L

901     GACAGCGAACAATCAGCACTTGCAACTGATCAGCTACGTGCACTCTACTCACAGGTTCGT
301      D  S  E  Q  S  A  L  A  T  D  Q  L  R  A  L  Y  S  Q  V  R

961     CCAAATGCTGTGGCTCTTGTAGATGCATTTAATTATACAGATCACTTTTTGGGATCTATA
321      P  N  A  V  A  L  V  D  A  F  N  Y  T  D  H  F  L  G  S  I

1021    CTTGGACGATATGATGGAAATGTCTATCCACATCTATATKAGGAAGCTTTCAAGGATCCT
341      L  G  R  Y  D  G  N  V  Y  P  H  L  Y  X  E  A  F  K  D  P

1081    TTGAATGAGACAGTTGTACCCGAAGGTTATGAGGAACACATTCGTCCACTTCTCAAGCAA
361      L  N  E  T  V  V  P  E  G  Y  E  E  H  I  R  P  L  L  K  Q

1141    CAGGTTAAAATTTCAAAATTGTGAATTAAAAGGTTTAAATTATTTCCAGGATATATCTAT
381      Q  V  K  I  S  K  L  *  I  K  R  F  K  L  F  P  G  Y  I  Y

1201    GTAAATTATATTTTCTATCCTATTATTGAATAATTTGACGTCCACGCATGGTTTTGGAGT
401      V  N  Y  I  F  Y  P  I  I  E  *  F  D  V  H  A  W  F  W  S

1261    CACTATTGTTTGATAAAGATATCATTGATATACTTGTCTAAAAAAAAAAAAAAAAAAAA
421      H  Y  C  L  I  K  I  S  L  I  Y  L  S  K  K  K  K  K  K
```

Figure 3.10. DNAMAN alignment of amino acid sequences of loblolly pine ACOX, arabidopsis ACOX, barleyACOX, and rat ACOX. The regions of identity are shaded in dark blue and regions of high homology are in light gray.


Loblolly
Arabidopsis
Barley
Rat
Consensus
l ygtmv vr v a ls a ia rysa r q e q
tqq lfplla aya fvg k y d l e
a i cr cgghgy s g p pec
t g a k g l
e r a
e l ah v f k
p q l

3.8 Tissue distribution of ACOX41 transcripts

Figure 3.11 shows differential accumulation of ACOX41 transcripts in different tissues of loblolly pine. There were very low levels of ACOX41 transcripts in the shoot pole, needle and root pole in loblolly pine when compared to ACOX41 transcript levels in the megagametophytes.

3.9 Changes in ACOX protein levels *in vivo*

To study the developmental profile of ACOX steady-state protein levels in loblolly pine seeds, a western blot analysis of mature, stratified and 2-12 DAI_{30} megagametophyte cell free extracts of the soluble protein fraction was performed. ACOX protein was undetected in extracts from mature and stratified seed (Figure 3.12). Detectable ACOX protein was observed following 4 DAI_{30} when there was a steady increase in protein amount. ACOX protein levels peaked 10 DAI_{30}, and then declined.

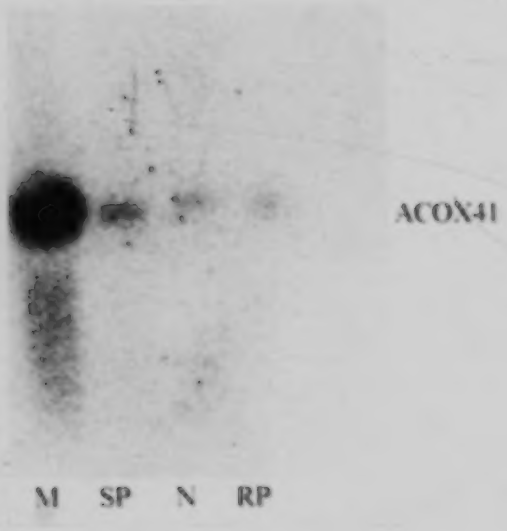


Figure 3.11 Accumulation of ACOX mRNA in different tissues of loblolly pine 10 DAI_{MG}. 10 µg of total RNA isolated from megagametophyte (M), shoot pole (SP), needle (N), and root pole (SP) were separated on formaldehyde-agarose gel and transferred to nylon membrane. Blots were hybridized with a $\alpha[^{32}P]$-radiorabelled ACOX41 cDNA probe, washed and exposed to X-ray film for 24 h at -80°C using an intensifying screen

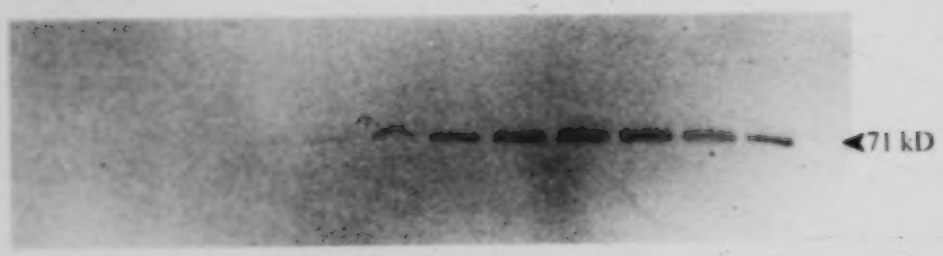


Figure 3.12. Immunoblot of ACOX steady state protein levels in megagametophytes following seed imbibition. Buffer-soluble protein extracts from megagametophytes from mature (M), stratified (S), and 2 through 12 DAI_{30} seeds were subjected to 12% SDS-PAGE, transferred to nitrocellulose, and probed with ACOX antiserum. For all lanes, 10 µg of protein was loaded

3.10 *De novo* ACOX protein synthesis

Immunoprecipitation was used to follow the synthesis of ACOX protein in vivo. Isolated megagametophytes were labeled for 3 h with [^{35}S]-methionine and buffer soluble proteins were extracted. Labeled cell free extracts were immunoselected with ACOX antiserum. A single polypeptide migrating to the same position as purified (71 kDa) was detected after 12% SDS-PAGE and fluorography. As seen in Figure 3.13, the rate of ACOX protein synthesis in mature and stratified seeds was low, but increased between 2 and 4 DAI_{30}. Maximum synthesis occurred 8 DAI_{30} and then declined.

3.11 Changes in ACOX transcripts during megagametophyte development

The accumulation of ACOX41 transcripts in the megagametophytes following seed imbibition was examined by northern blotting. Total RNA extracted from megagametophytes isolated from mature, stratified, and seeds following imbibition at 30^{0}C, was separated by formaldehyde agarose gel electrophoresis. The RNA was then passively transferred to a nylon membrane, and probed with α[^{32}P]-radiolabelled ACOX cDNA. Low levels of ACOX41 transcripts were detected megagametophytes from mature, stratified and 2 DAI_{30} seeds. Transcript levels increased 4 DAI_{30} and this increase continued to 10 DAI_{30}, after which transcript levels slightly decreased.

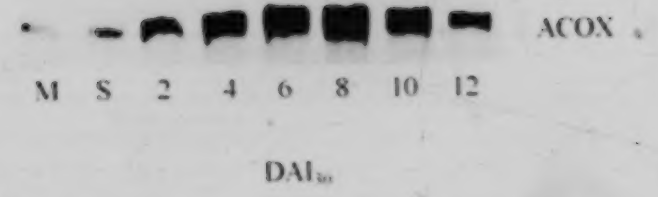


Figure 3.13. De novo protein synthesis of ACOX protein following seed imbibition. Isolated megagametophytes from mature (M), stratified (S), and 2 through 12 DAI_{30} seeds were labelled with [^{35}S]-methionine for 3 h at 30°C. For all stages ACOX was immunoselected with ACOX antiserum from total buffer soluble protein extracts which were adjusted such that the counts for each stage were equal (300,000 dpm). Immunoselected ACOX was then subjected to 12% SDS-PAGE and fluorography.

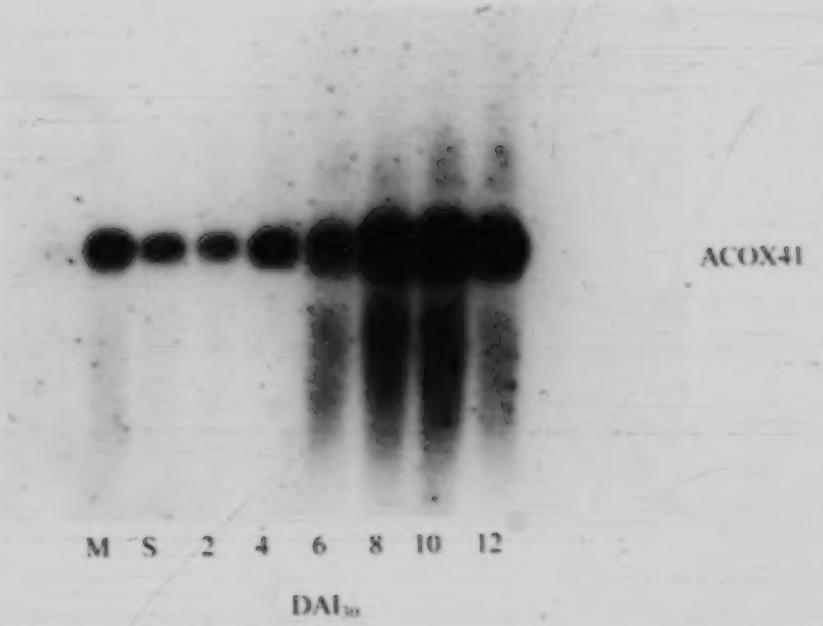


Figure 3.14. Accumulation of ACOX mRNA in megagametophytes following imbibition. 10 µg of total RNA isolated from mature (M), stratified (S), and 2 through 12 DAI_{30} seeds were separated on formaldehyde-agarose gel and transferred to nylon membrane. Blots were hybridized with a $\alpha[^{32}P]$-radiorabelled ACOX41 cDNA probe, washed and exposed to X-ray film for 24 h at -80°C using an intensifying screen.

3.12 Effect of embryo removal on *in vivo* protein synthesis in the developing megagametophytes

To examine the effect of the embryo removal on protein synthesis, megagametophytes were isolated from mature seeds, and from megagametophytes cultured up to 6 DAI_{30} *in vitro* in the presence and absence of the embryo. Megagametophytes were taken incubated at 30^0C for 3 h with [^{35}S]-methionine. Buffer soluble proteins were then extracted and analyzed by one-dimensional SDS-PAGE followed by fluorography (Figure 3.15). Buffer soluble proteins extracted from 4 DAI_{30} were analyzed further using two-dimensional IEF/SDS-PAGE followed by fluorography (Figure 3.16). An examination of Figures 3.15 and 3.16 showed that removal of the embryo caused a change in the pattern of protein synthesis occurring in the megagametophyte. For example, SDS-PAGE revealed that proteins in the 28 kDa region of the gel were only synthesized in the megagametophyte when the embryo was present, in contrast proteins in the 32 and 38 kDa region of the gel were only synthesized when the embryo was absent. In addition to the observed differences, some proteins were synthesized regardless of the presence or absence of the embryo, for example those in the 70-92 kDa region of the gel.

In Figure 3.16, examples of proteins that are synthesized constitutively are shown in circles whereas examples of those that differentially synthesized are shown in squares. It is noteworthy that, even those proteins that were constitutively synthesized are synthesized to a lesser extent in the absence of the

embryo. In relation to this, quantification of ^{35}S-metionine incorporation into protein was performed by TCA precipitation, and the results showed that there was approximately 80% reduction in incorporation in megagametophytes that were cultured *in vitro* in the absence of embryos. These differences occurred to be over a wide range of pIs and molecular weights.

3.13 Effect of embryo removal on TAG concentrations in the megagametophyte during germination and post germinative growth

Quantitative changes in TAG reserves of megagametophytes cultured at 30^{0}C, with or without the embryos are shown in Figure 3.17. In megagametophytes cultured *in vitro* with the embryo, TAGs remained constant until 3 DAI_{30}, when there was a rapid decrease of TAG levels. By 10 DAI_{30}, TAGs were depleted by 83%. In contrast, in megagametophytes cultured *in vitro* without embryos showed TAG breakdown during the first 3 days of imbibition, after 3 DAI_{30} however, TAG breakdown declined, remaining fairly constant through 10 DAI_{30}.

Figure 3.15. One-dimensional gel electrophoresis fluorographs of [^{35}S]-methionine-labeled soluble proteins extracted from megagametophytes of seeds taken from mature, 2, 4, and 6 DAI_{30}, germinated with (+) or without (-) the embryo. For each lane 15 000 dpm were loaded. Numerical values to the left of the gel are molecular mass in kDa of methylated protein standards.

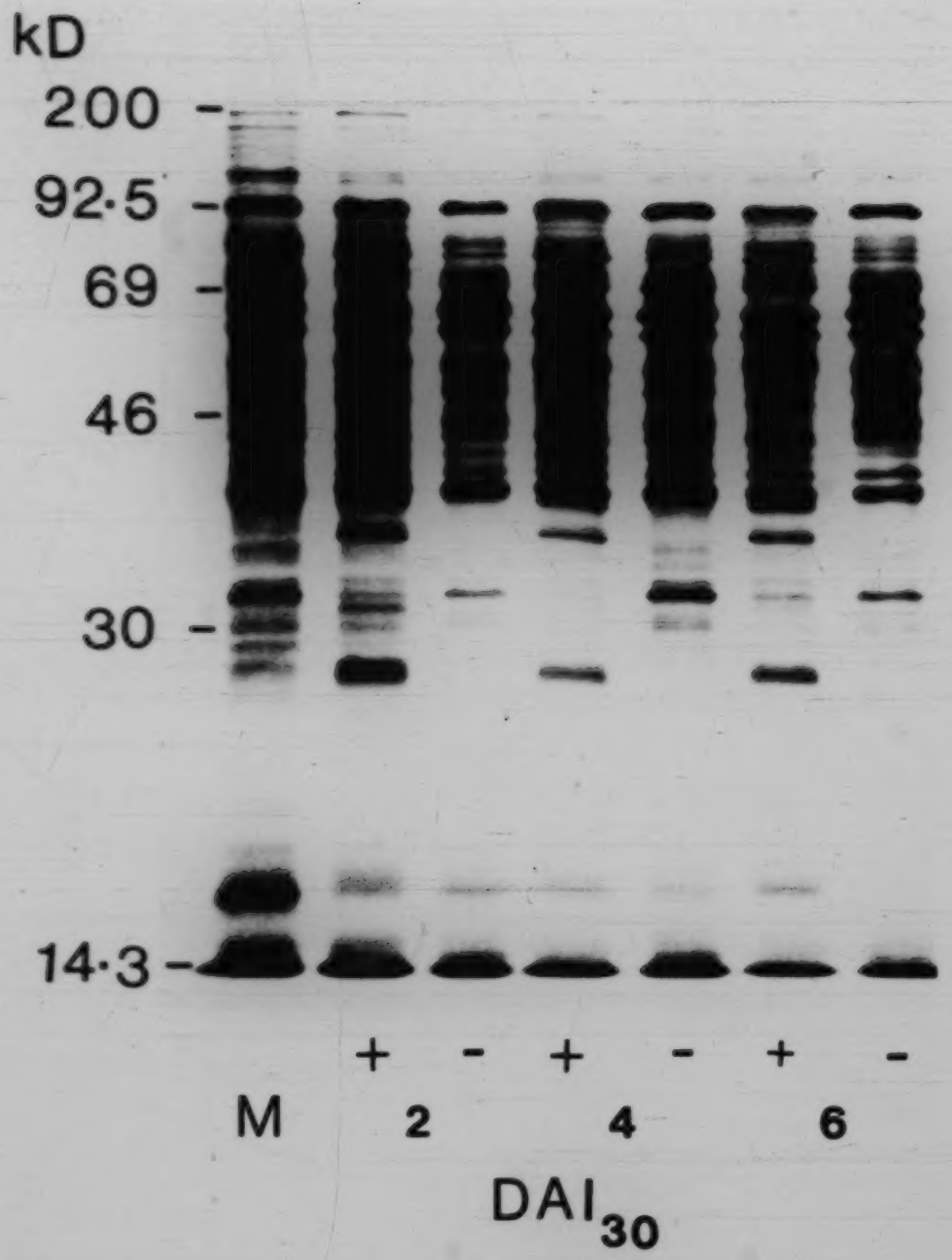

kD
200
92·5
69
46
30
14·3
+
-
+
-
+
-
M
2
4
6
DAI30

Figure 3.16. Two-dimensional gel electrophoresis fluorographs of [^{35}S] methionine-labeled soluble proteins extracted from megagametophytes of seeds taken from 4 DAI_{30}, germinated with the embryo (A) or without the embryo (B). For each gel 25 000 dpm were loaded. Range of pH for the first dimension (pI 4.5-8.5) is indicated at the top. Numerical values to the left are molecular mass in kDa of methylated protein standards run in the second dimension; O, represents representative proteins synthesized constitutively in both megagametophytes from seeds germinated with or without the embryo; □, representative proteins synthesized differentially in megagametophytes from seeds germinated with or without the embryo.

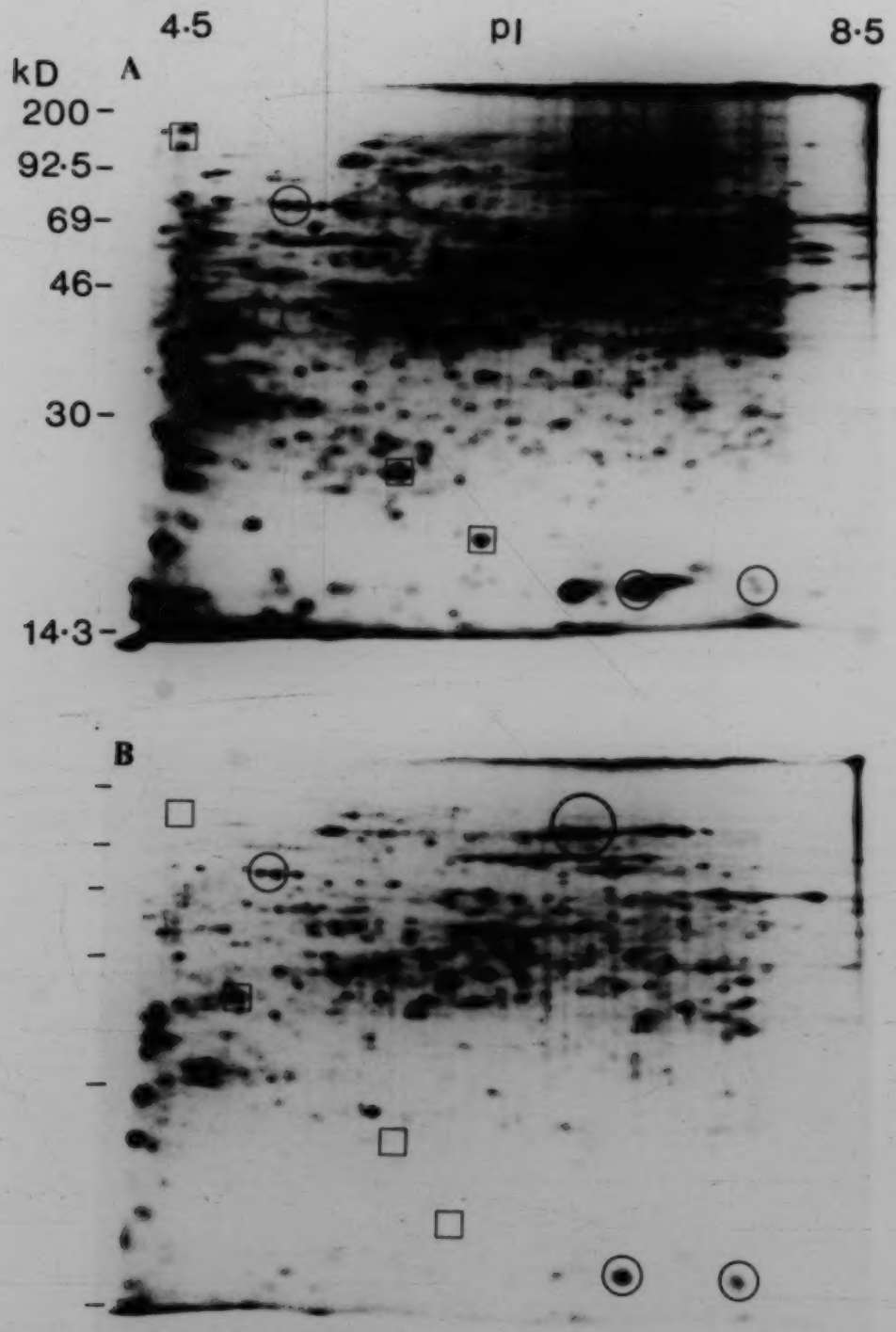
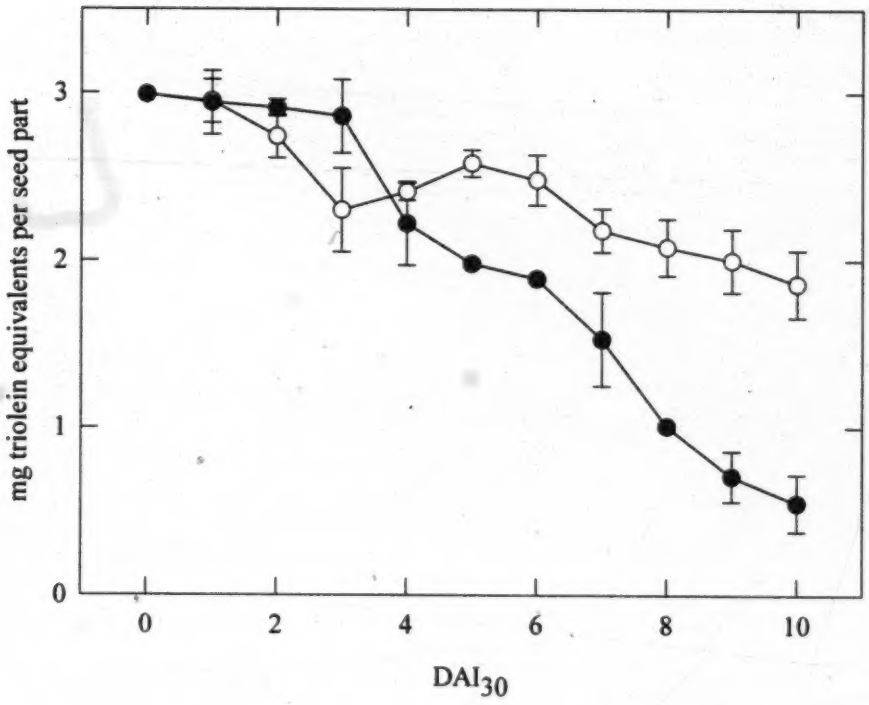

4·5
pI
8·5
kD
A
200
92·5
69
46
30
14·3
B

Figure 3.17. Quantitative changes of TAG levels in megagametophytes cultured *in vitro* with (closed circles) or without (open circles) the embryo. Each data point is a mean of three independent biological replicates each assayed in triplicate.


mg triolein equivalents per seed part
0
1
2
3
0
2
4
6
8
10
DAI30

3.14 Effect of embryo removal on carbohydrate levels in the megagametophyte during germination and post germinative growth

Quantitative changes of soluble carbohydrates in megagametophytes that were cultured *in vitro* with or without the embryo are shown in Figure 3.18. In both cases, the soluble carbohydrate levels decreased rapidly following imbibition until 2 DAI_{30}. In megagametophytes cultured *in vitro* with the embryo this decline continued at a slower rate until 10 DAI_{30}. This was in contrast to, megagametophytes that were cultured *in vitro* without embryos where there was an increase in soluble carbohydrate level until 6 DAI_{30}. Changes in total insoluble carbohydrates are shown in Figure 3.19. Insoluble carbohydrate levels were approximately 10 times lower than the soluble carbohydrates in the mature megagametophyte. Following seed imbibition, the insoluble carbohydrate levels remained fairly constant in the megagametophytes cultured *in vitro* with embryos. The insoluble carbohydrate levels in the megagametophytes cultured *in vitro* without the embryos, decreased slightly until 2 DAI_{30}, following which, the levels started to increase to a maximum at 8 DAI_{30}. At 8 DAI_{30} there was approximately twice as much insoluble carbohydrate in megagametophyte cultures in the absence of the embryo.

Figure 3.18. Quantitative changes of soluble carbohydrate levels megagametophytes cultured *in vitro* with (closed circles) or without (open circles) the embryo. Each data point is a mean of three independent biological replicates each assayed in triplicate.

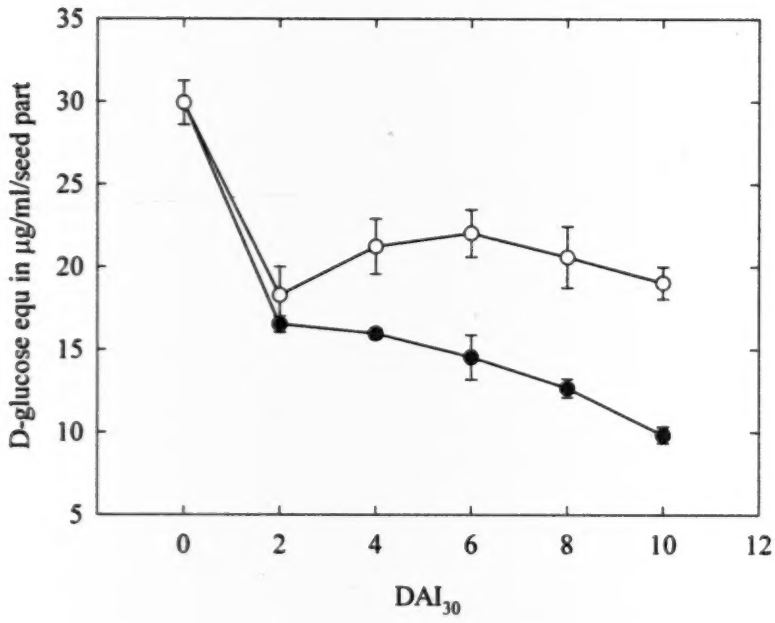
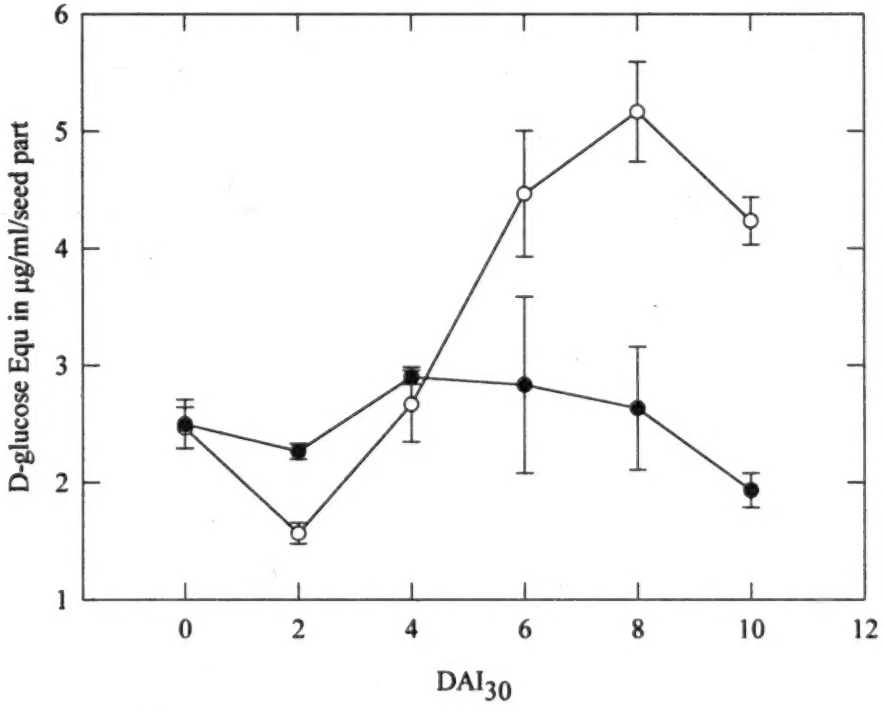

35
30
25
20
15
10
5
D-glucose equ in µg/ml/seed part
0
2
4
6
8
10
12
DAI30

Figure 3.19. Quantitative changes of insoluble carbohydrate levels in megagametophytes cultured *in vitro* with (closed circles) or without (open circles) the embryo. Each data point is a mean of three independent biological replicates each assayed in triplicate.


D-glucose Equ in µg/ml/seed part
6
5
4
3
2
1
0
2
4
6
8
10
12
DAI30

3.15 Effect of embryo removal on ACOX and CAT activities

When the megagametophytes were cultured *in vitro* in the presence of the embryos as seen in Figure 3.20, ACOX activity followed the same trend as that seen in megagametophytes from normally developing seeds (Figure 3.1). In contrast, much lower ACOX activity was observed in megagametophytes cultured *in vitro* in the absence of the embryo (Figure 3.20). Similar results were obtained for the activity of CAT when megagametophytes were cultured *in vitro* as seen in Figure 3.21. In this case, very low levels of CAT activity were observed in megagametophytes that were cultured *in vitro* without embryos.

3.16 Effect of soluble carbohydrates on ACOX activity *in vitro*

Since soluble carbohydrate accumulation (Figure3.18), and low levels of ACOX activity (Figure 3.20) were observed in cell-free extracts of megagametophytes cultured *in vitro* without embryos, the effect of specific soluble carbohydrates on ACOX activity in cell-free extracts was determined. Addition of sucrose, glucose or fructose to the reaction mixture did not affect ACOX activity in cell-free extracts of megagametophytes taken 10 DAI_{30}.

Figure 3.20. Effect of embryo removal on ACOX activity in megagametophytes cultured *in vitro* with (closed circles) or without (open circles) embryos. Each data point is a mean of three independent determinations.

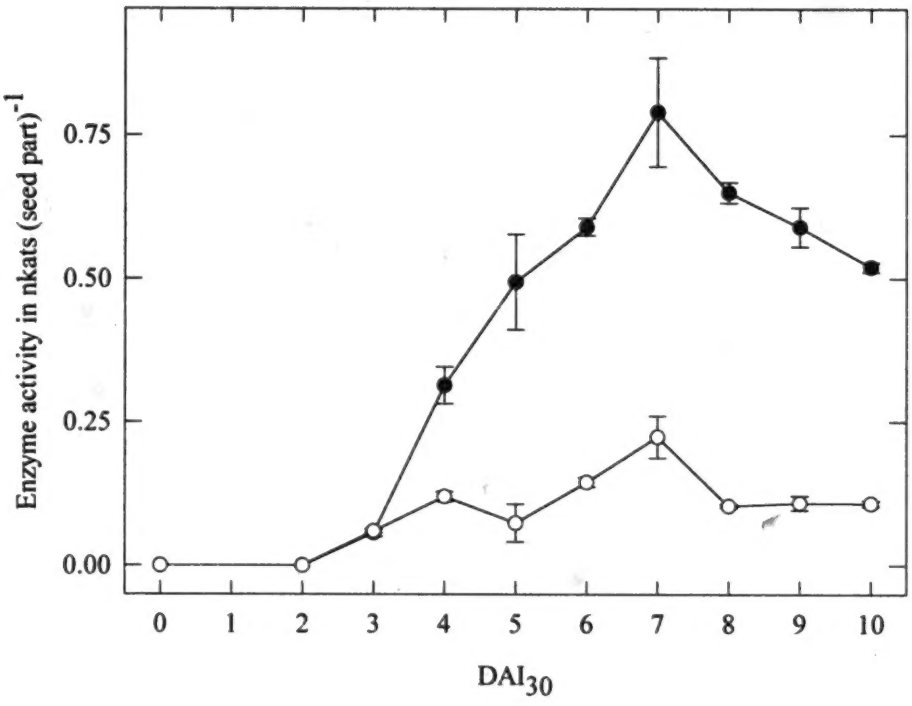
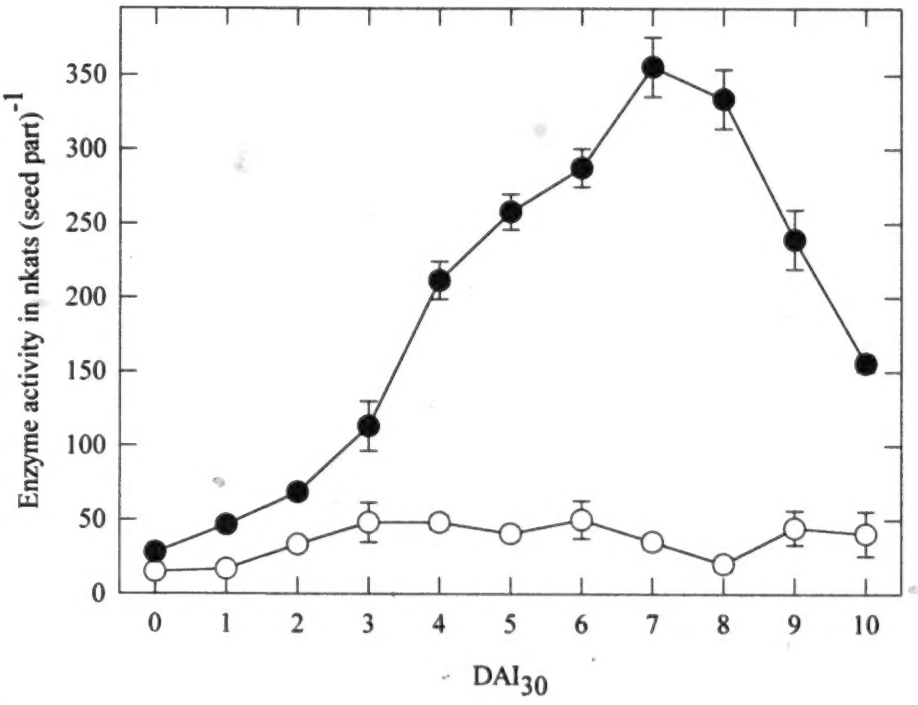

Enzyme activity in nkats (seed part)$^{-1}$
0.00
0.25
0.50
0.75
0
1
2
3
4
5
6
7
8
9
10
DAI_{30}

Figure 3.21. Effect of embryo removal on CAT activity in megagametophytes cultured *in vitro* with (closed circles) or without (open circles) embryos. Each data point is a mean of three independent determinations.


Enzyme activity in nkats (seed part)$^{-1}$
350
300
250
200
150
100
50
0
0
1
2
3
4
5
6
7
8
9
10
DAI$_{30}$

3.17 Effect of embryo removal on the accumulation of ACOX protein in megagametophytes cultured *in vitro*

Using a Western blot analysis, total ACOX protein levels in cell-free extract taken from megagametophytes cultured *in vitro* in the presence or absence of the embryo were compared (Figure 3.23). With the possible exception of megagametophytes taken 2 DAI_{30}, there was always less ACOX protein in megagametophytes cultured without the embryo taken at the stages used in the experiment.

3.18 Effect of embryo removal on the accumulation of ACOX transcripts in megagametophytes cultured *in vitro*

Total RNA was extracted from megagametophytes incubated with and without the embryo and the presence of ACOX41 transcripts was determined using a northern blot analysis (Figure 3.24). Initially the accumulation of ACOX41 transcripts in the megagametophytes cultured without the embryos was higher, however after 4 DAI_{30}, the level of ACOX41 transcripts in megagametophytes cultured without embryos declined rapidly, while ACOX 41 transcripts in megagametophytes cultured with embryos present increased to a maximum at 8 DAI_{30}, and then declined slightly.

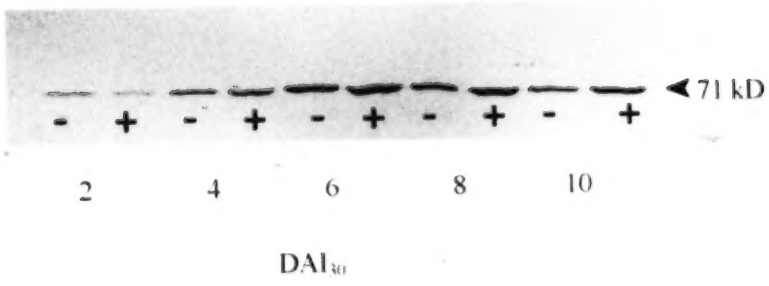


Figure 3.23. Immunoblot of ACOX steady state protein levels in megagametophytes cultured in vitro in the presence (+) or absence (-) of the embryo. Buffer-soluble protein extracts isolated from megagametophytes taken from 2 through10 DAI_{30} seeds were subjected to 12% SDS-PAGE, transferred to nitrocellulose, and probed with ACOX antiserum. For all lanes, 10 µg of protein was loaded.

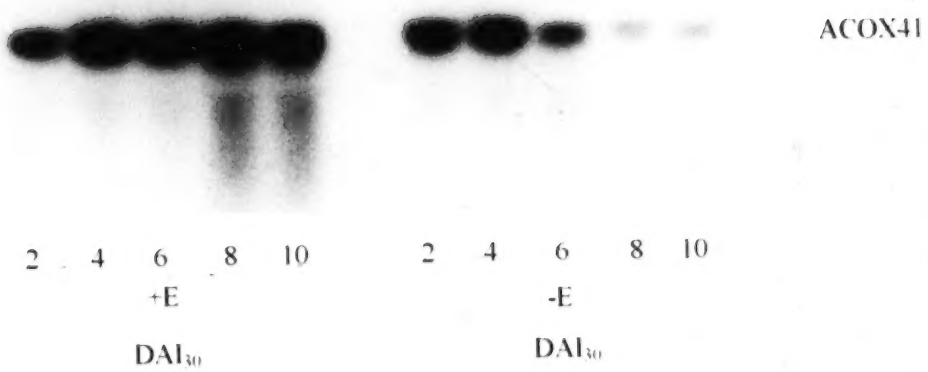


Figure 3.24. Accumulation of ACOX mRNA in megagametophytes cultured in vitro in the prence (+E) or absence (-E) of the embryo. 10 µg of total RNA isolated from megagametophytes taken from 2 through 10 DAI_{30} seeds were separated on formaldehyde agarose gel and transferred to nylon membrane. Blots were hybridized with a $\alpha[^{32}P]$-radiorabelled ACOX41 cDNA probe, washed and exposed to X-ray film for 24 h at -80°C using an intensifying screen.

4. Discussion

4.1 Purification and characterization of ACOX

The enzyme ACOX has been purified from a variety of species including cucumber (Kirsch et al., 1986), pumpkin (Hayashi et al., 1998), rat (Osumi et al., 1980), yeast (Luo et al., 1996 and Wayne and Rachubinski 1987), and human (Baumgart et al., 1996). To my knowledge, this thesis presents the first purification of this enzyme from a conifer.

Acetone was used in the initial step of the purification to remove most of the lipids present in loblolly pine crude extracts that would normally interfere with the enzyme assays. The homogenate from this step was taken as the crude extract. Because ACOX was a not heat labile it was possible to use a heat treatment to eliminate other more labile proteins, thereby purifying the enzyme 2.5-fold (Table 1). A similar approach was taken during the initial stages of the purification of another loblolly pine protein arginase (King, 1998) and a cucumber ACOX (Kirsch et al., 1986). While ammonium sulphate fractionation to 45% effectively concentrated the enzyme from the large volume supernatant and provided a further purification of 22-fold, this step resulted in a 43% decrease in yield. To improve the yield, a higher ammonium sulphate saturation (55%) was attempted. This approach resulted in higher yield, but, enzyme specific activity decreased to a level that was deemed too low for subsequent purification steps; it

should be noted that in this purification the strategy to prefer higher specific activity to yield was adopted. ACOX protein has been shown to be hydrophobic. Because of this, Phenyl-Sepharose chromatography was used to separate ACOX from other proteins that were less hydrophobic (Hooks et al., 1996, Kirsch et al., 1986). Chromatography on the phenyl-sepharose column resulted in its separation from most other proteins (Figure 3.2). Indeed, after this step, SDS-PAGE showed that the fractions with ACOX activity contained only two bands (Figure 3.5 lane D). Hydroxyapatite chromatography that followed, which selectively adsorbs proteins and allowing their release at higher salt concentrations (Scopes, 1982), served to greatly enrich ACOX specific activity further (Figure 3.3). Finally, fractionation by molecular sieving (Figure 3.4) produced a homogeneous enzyme with a molecular mass of 150 kDa, which comprised two identical subunits of 71 kDa.

Loblolly pine ACOX appears to be similar to the cucumber enzyme, which is a homodimer with 2 subunits of molecular mass of 72 kDa each (Kirsch et al., 1986). Different oligomerization, however, has been reported for ACOX from other organisms. For example the ACOX from *Candida tropicalis* is octomeric with a subunit molecular mass of 74 kDa (Okazaki et al., 1987; Shimizu et al., 1979). The rat liver enzyme on the other hand is reported to be a heterotrimer with subunit molecular masses of 75.5, 50 and 19 kDa (Osumi et al., 1980). In contrast, the human liver ACOX is a monomeric enzyme with a molecular mass of 70 kDa (Vanhove, 1993).

Plants contain ACOX isozymes that are active on short, medium and long-chain acyl-CoAs (Hooks et al., 1996). It is likely that the ACOX purification reported here was for the enzyme that exhibited preference for long chain acyl-CoAs; there are several reasons for this. Palmitoyl-CoA was used as the substrate for ACOX activity during the protein purification. The purified protein was a homodimer with a subunit molecular mass of approximately 71 kDa, which is similar to that reported for cucumber (Kirsch et al., 1986) and pumpkin (Hayashi et al., 1998) long-chain ACOX subunits; which showed molecular masses of 72 kDa and 73 kDa respectively. Medium and short-chain ACOX isoforms from maize have been shown to have different subunit molecular masses; the medium-chain isoform is a monomeric enzyme of 62 kDa, whereas the short-chain isoform is a homotetrameric enzyme of a molecular mass of 15 kDa (Hooks et al., 1996). Finally, short-chain acyl-CoA oxidases from pumpkin and arabidopsis were shown to be homotetramers with a subunit molecular mass of 47 kDa and a native molecular mass of 180 kDa (Hayashi et al., 1999).

4.2 Isolation of a cDNA that encodes ACOX

Although cDNAs encoding ACOX have been isolated from several higher plant species including arabidopsis (*Arabidopsis thaliana*) (Hooks et al., 1999), and pumpkin (Hayashi et al., 1998), the isolation of an ACOX cDNA from loblolly pine reported here is a first for a conifer.

Immunoscreening of a loblolly pine megagametophyte λ ZAP cDNA library (Mullen and Gifford 1997) resulted in isolation of several clones whose sequence corresponded to known ACOX cDNAs. However, when the amino acid sequence deduced from the longest cDNA obtained was compared to deduced amino acid sequences from full length ACOX cDNAs encoding subunits of similar size (approx 71 kDa) from other plant and animal sources (Hayashi et al., 1998; Murray and Rachubinski 1987; Dmochowska et al., 1990; Hooks et al., 1999; Fan et al., 1996 and Baumgart et al., 1996), it was determined that the loblolly pine ACOX cDNA was not full length; approximately 500 base pairs from the 5' end of the cDNA were missing. To obtain a full-length ACOX cDNA it will be necessary to screen the cDNA library in a more rigorous fashion. Alternatively, it may be possible to generate the 5' end of the ACOX cDNA using a rapid amplification of cDNA ends (RACE) approach.

The loblolly pine ACOX cDNA sequence obtained contained an open reading frame of 1164 base pairs. The C-terminal end of this open reading frame contained a SKL motif. The majority of plant and animal peroxisomal proteins are targeted to the matrix of peroxisomes by a PTSI type targeting motif. This C-terminal motif is a tripeptide comprising Ser-Lys-Leu (SKL) or its variants (Brickner et al., 1997, Gietl, 1996, Rachubinski and Subramani, 1995, Olsen et al., 1993). Loblolly pine ACOX appears to be similar in this regard. Arabidopsis ACOX also has a PTSI targeting motif (Hooks et al., 1999). In contrast, pumpkin ACOX has a PTS2-type targeting motif (Hayashi et al., 1998), which comprises a N-terminal nonapeptide (R/K)(L/V/I)$(X)_5$(H/Q)(L/A). Also, ACOX from several

species of yeast posses both N-terminal and internal targeting sequences (Swinkels et al. 1991, Small et al. 1988; Kamiryo et al. 1989).

Loblolly pine ACOX cDNA also contained a FAD binding motif, CGGHGY (Hooks et al.1999; Dubourdieu and Fox 1977). This is not surprising given the fact that ACOX is a FAD dependent enzyme (Alberts et al. 1994).

4.3 ACOX synthesis and expression: changes occurring during germination and early seedling growth

4.3.1 Tissue specificity

Western blot analysis of soluble protein extracts of 10 DAI_{30} material showed that ACOX protein was predominantly in the megagametophyte and only very small amounts were detected in the shoot pole (Figure 3.8); this result was in agreement with preliminary ACOX enzyme data. The presence of ACOX enzyme activity and protein in cell free extracts of megagametophytes is not surprising given that TAG breakdown via β-oxidation and the glyoxylate cycle is well documented in megagametophytes of loblolly pine and other conifers (Ching, 1970; Mullen and Gifford, 1995a, 1995b). What is surprising is the very low level of ACOX activity and protein detected in cell free extracts of the developing seedling, given the fact that TAGs comprise 40% of the mature embryo's dry weight (Stone and Gifford, 1999), and are broken down during early

seedling growth. Cell free extracts of loblolly pine seedlings also contain very low levels of ICL and MS activity and protein (Mullen and Gifford, 1995a; Mullen and Gifford, 1995b), indicating that there may not be an active glyoxylate cycle in these tissues. Low or undetectable levels of glyoxylate cycle enzymes have also been reported in cell free extracts of seedlings of several other species of pine including ponderosa pine (*Pinus ponderosa* Laws)(Ching, 1970) and sugar pine (*Pinus lambertiana*) (Noland and Murphy, 1984). Because of these observations of glyoxylate cycle enzymes, it has been argued that TAG breakdown during pine early seedling growth may occur through the glyoxysomal β-oxidation spiral, releasing energy through the mitochondrial Krebs cycle (Stone and Gifford, 1999) and not via the glyoxylate cycle. In the light of data presented in this thesis, i.e. the detected low level of ACOX gene expression in the seedling, this scenario seems unlikely. An alternate scenario is that loblolly pine seedlings do contain a β-oxidation spiral coupled to a glyoxylate cycle, but that these processes operate at very low rates. In this regard, the rate of TAG breakdown in the seedling is significantly slower than in the megagametophyte (Stone and Gifford, 1999). The exact nature of TAG breakdown in pine during early seedling growth has yet to be determined.

4.3.2 Temporal changes following seed imbibition

Results of studies of angiosperm genes encoding glyoxysomal enzymes such as ACOX and ICL led to the conclusion that these genes are controlled primarily at the level of mRNA (Hooks et al., 1999; Hayashi et al., 1998; Turley and Trelease, 1990). A similar conclusion was reached for loblolly pine ICL gene expression (Mullen and Gifford, 1997). It appears that regulation of ACOX gene expression in loblolly pine megagametophytes is similar in this regard; there is a correlation between temporal accumulation of ACOX mRNA, steady-state protein and enzyme activity and ACOX protein synthesis at all stages of megagametophyte development examined. ACOX gene expression is also regulated at the level of mRNA in *Saccharomyces cerevisiae* (Luo et al., 1996; Stanway et al., 1995; Wang et al., 1994; Wang et al., 1992; and Dmochowska et al., 1990) and *Candida tropicalis* (Fujiki et al., 1986).

The temporal correlation mentioned above for loblolly pine ACOX mRNA, steady-state protein and enzyme activity during the later stages of early seedling growth is in contrast to that observed for megagametophyte ICL gene expression (Mullen and Gifford, 1997). In this case, steady state levels of ICL mRNA and protein remain high in megagametophytes of seeds 10-12 DAI_{30} whereas ICL activity and the rate of *in vivo* synthesis of ICL protein declined rapidly. Mullen and Gifford (1997) concluded that in addition to regulation at the level of mRNA, loblolly pine ICL gene expression appeared to be regulated at the translation or post-translation level. Similar observations have been made for

rape seed (*Brassica napus*) seedling ICL and MS (Ettinger and Harada, 1990). The regulation of loblolly pine ACOX gene expression does not appear to be similar in this regard; regulation appears to be solely at the level of mRNA.

Interestingly, although ACOX mRNA and *in vivo* protein synthesis was present in the megagametophyte of mature and stratified seed, ACOX enzyme activity was not detected in the same cell-free extracts. A similar observation was made in relation to the lack of ICL activity in mature and stratified seeds of loblolly pine (Mullen and Gifford, 1997) and in mature seeds of cucumber (Frevert et al., 1980), sunflower (Fusseder et al., 1984; Theimer, 1976) and cotton (Choinski and Trelease, 1978). In these cases the lack of ICL enzyme activity was attributed to the presence of an endogenous enzyme inhibitor present only in mature and stratified seed. Whether or not this observation holds true for loblolly pine ACOX enzyme has yet to be determined.

4.4. ACOX synthesis and expression: the relationship between the megagametophyte and embryo

4.4.1 TAG breakdown

The breakdown of the TAG reserve in the megagametophyte occurs primarily during the early seedling growth phase of seed development (Stone and Gifford, 1999). Since a large soluble carbohydrate pool does not accumulate in

the megagametophyte as TAGs are broken down, Stone and Gifford (1999) speculated that the soluble carbohydrates produced in this way are rapidly exported to the developing seedling; support for this was a large increase in the soluble carbohydrate pool of the seedling that correlated with TAG breakdown in the megagametophyte. This is different to that observed for the angiosperm oil seed castor bean (*Ricinus communis* L.) (Huang and Beevers, 1974) where a large increase in the soluble carbohydrate pool accumulated in the endosperm as TAG breakdown occurred. However, a similar correlation between TAG losses in the megagametophyte and gains in soluble carbohydrates in the seedling has been reported for Douglas fir (*Pseudotsuga menziesii* Franco) (Ching, 1966), Taiwan red pine (*Pinus taiwanensis* Hayata) (Kao, 1973), and Chinese fir (*Cunninghamia lanceolata* [Lamb] Hook) (Kao, 1973). In relation to this, a similar observation was made for loblolly pine amino acid export to the developing seedling during storage protein breakdown in the megagametophyte (King and Gifford, 1997). These conifer studies demonstrate that there is a dynamic relationship between megagametophyte and seedling of loblolly pine with respect to the transport of carbohydrates and amino acids. With regard to TAG breakdown, the results presented in this thesis provide further evidence for this dynamic relationship. Removal of the embryo completely altered the rate of TAG breakdown in the megagametophyte. While there was an initial rapid decrease in TAG levels in megagametophytes, this decrease stopped 2 DAI_{30} and by 3 DAI_{30} the TAG levels remained fairly constant through 12 DAI_{30} (Figure 3.17). Similarly, when the embryo was removed, there was an increase of soluble carbohydrates followed by

a large increase in insoluble carbohydrates. In the absence of the sink, it appears that soluble carbohydrates are converted into an insoluble carbohydrate form in order to effectively decrease the soluble carbohydrate pool so that TAG breakdown is maintained. In this regard, insoluble carbohydrate pool acts as a sink.

There are two major mechanisms by which the embryo could impart control on reserve breakdown: 1) to act as a sink, similar to what happens in cucumber where Davies and Chapman (1979a, 1979b) showed that removal of embryonic axis caused feedback inhibition that decreased both protein and lipid degradation, and 2) to secrete signal molecules that would then regulate reserve breakdown. This second case is common in cereals where hormones like gibberellins are secreted by embryonic axis to induce *de novo* synthesis of α-amylase in the aleurone layer. The synthesized α-amylase is then secreted to the endosperm where it is involved in the starch breakdown (Jacobsen et al., 1995, Bewley and Black 1994). In addition Yu et al. (1996) further demonstrated that both gibberellins and sugars serve as regulatory signals to directly or indirectly control the expression of α-amylase genes and metabolic activities, which would in turn control the rate of starch hydrolysis in the scutellum and the endosperm. The involvement of hormones in reserve breakdown was also demonstrated in castor bean where Gifford et al. (1984) showed that gibberellins played a role in initial breakdown of storage reserves. More studies in germinating castor bean suggested that hormones were responsible for transcriptional control of glyoxylate cycle enzymes, because exogenous application of gibberellins increased the

accumulation of transcripts of ICL (Martin et al., 1984) and MS (Rodriguez et al., 1987) and ABA inhibited the accumulation of these transcripts in both cases. In the case of loblolly pine, the involvement of a signal molecule is unlikely because lipid breakdown was observed in complete absence of the embryo. It appears that reserve breakdown in loblolly pine is controlled by accumulation of reserve breakdown products in storage tissues as was the case in cucumber (Davies and Chapman 1979a).

The mechanism by which breakdown products inhibit reserve breakdown varies. For example breakdown products can reduce activities of enzymes involved in reserve breakdown by product inhibition at the biochemical level. This was the case for castor bean TAG breakdown where it was shown that the accumulation of higher levels of sugars caused by the removal of embryonic axis reduced the activities of glyoxysomal enzymes (Huang and Beevers 1974; Lado et al., 1968). In this particular case it was shown that the application of exogenous glucose suppressed the activity of isocitrate lyse in cell free extracts of the endosperm (Lado et al., 1968). Lado et al. (1968) further showed that glucose application to cell free extracts of squash cotyledons taken during germination and early seeding growth inhibited isocitrate lyase. This does not apply to loblolly pine because the addition of glucose, fructose or sucrose did not affect ACOX activity in megagametophyte cell free extracts. Another mechanism by which accumulation of breakdown products could inhibit enzymes involved in storage reserve breakdown is by catabolite repression. This was shown in cucumber by Graham et al. (1994), who demonstrated that addition of glucose,

fructose and raffinose to a cell suspension culture repressed MS and ICL gene expression. This latter mechanism appears to be more likely for the loblolly pine ACOX regulation. This is because *in vivo* protein labeling of megagametophytes that had been cultured *in vitro* in the absence of embryos, was reduced (Figures 3.16 and 3.17) quantitatively and qualitatively; i.e. total incorporation of label into proteins was reduced, as was the labeling of specific proteins. This was also demonstrated for ACOX where western and northern blot analyses showed that ACOX protein and mRNA levels were repressed by the removal of the embryo (Figure 3.23 and 3.24).

4.4.2 ACOX gene expression and enzyme activity

The first and rate-limiting step of β-oxidation is catalyzed by ACOX, which makes it a very important enzyme in the process of TAG breakdown (Baumgart, et al. 1996; Luo, et al. 1996; Wang, et al. 1992; Kindl, 1987; Wayne and Rachubinski, 1987; Kirsch, et al. 1986; Yukio et al. 1986; Yukio and Lazarow, 1985; and Osumi and Hashimoto, 1979). The reaction that ACOX catalyzes is coupled to the reaction catalyzed by CAT. ACOX is developmentally regulated as demonstrated by the temporal changes in enzyme activity, protein and mRNA levels. This has been shown to be the case in other loblolly pine glyoxysomal enzymes including CAT (Mullen and Gifford 1995a, 1995b). Both the activities of ACOX and CAT were greatly reduced in cell free extracts of

megagametophytes cultured *in vitro* in the absence of the embryo (Figures 3.20 and 3.21), Western and Northern analyses showed that ACOX was regulated at mRNA level. This because steady state protein and mRNA levels were also greatly reduced in cell free extracts of megagametophytes cultured in vitro in the absence of the embryo (Figures 3.23 and 3.24).

Studies in yeast have shown that ACOX gene expression is under the regulation of carbon catabolite repression, where hexose sugars are the major players that bring about this control. It has also been shown that ACOX is repressed by hexose sugars and activated by oleic acid, and this regulation of ACOX in yeast was brought about by complex interactions between *trans*-acting factors and *cis*-elements in the promoter of ACOX gene (Luo et al.1996; Stanway et al. 1995; Wang et al.1994; Wang et al. 1992; and Dmochowska et al. 1990). Graham et al. (1992) showed that the gene expression of the glyoxysomal enzymes MS and ICL was repressed by sucrose in cucumber suspension culture cells. Graham et al., (1994) also showed that glucose, fructose and raffinose were as effective as sucrose in repressing ICL and MS gene expression. The same investigator proposed that the mechanism of this regulation is directly associated with hexose phosphorylation by hexokinases, because addition of 3-methylglucose, an analog of glucose which is not phosphorylated did not repress MS and ICL gene expression.

Based on these observations, it is possible that loblolly pine ACOX gene expression is also regulated by catabolite repression; given that mRNA

accumulation is much lower in megagametophytes that accumulate soluble carbohydrates.

As an interesting corollary, one can argue that the low levels of ACOX mRNA and protein observed in the seedling are also due to soluble carbohydrate repression of ACOX gene expression. This is because the seedling is actively importing soluble carbohydrates including sucrose from the megagametophyte; in the cotyledons (Stone and Gifford 1999), the sucrose can be readily converted to glucose and fructose, which can affect ACOX gene expression.

In conclusion, the data presented in this thesis supports the hypothesis that the developing seedling controls TAG breakdown in the megagametophyte. This control is primarily at the level of mRNA synthesis.

4.5 Future studies

In this thesis, it has been demonstrated that TAG breakdown in loblolly pine megagametophyte is regulated by the seedling during its early growth. The major enzyme (ACOX) that controls the flux of carbon in the β-oxidation spiral is regulated by the seedling and this regulation is at the level of mRNA. However, the exact nature of this regulation has yet to be elucidated. I have suggested that ACOX gene expression is regulated by carbon catabolite repression. This needs to be studied further. Using a cell suspension culture system, it would be possible to determine which metabolites repress or derepress loblolly pine ACOX gene

expression. Further studies of how loblolly pine ACOX is regulated will require full length cDNA and genomic DNA sequences.

With the sequences at hand, the mechanism of ACOX regulation could then be studied by promoter analysis. Functional analysis of the promoter region of the gene and mobility shift assays would give the gene sequences that are responsible for this regulation. It has been shown that glucose catabolite repression is directly associated with hexose phoshorylation by hexokinases in cucumber (Graham et al., 1994) and in yeast (Rose et al., 1991; and Ma et al., 1989a; 1989b). It would be interesting to find out if the same mechanism applies in loblolly pine.

In conclusion, this study and future studies, which will examine how the metabolic status of a cell regulates glyoxylate cycle gene expression, will extend our knowledge of conifer seed biology. For example, overexpression of ACOX in megagametophytes could increase TAG breakdown in this tissue which in turn could result in increased rates of germination. This information may prove useful in the forest industry to overcome problems associated with germination and seedling establishment.

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